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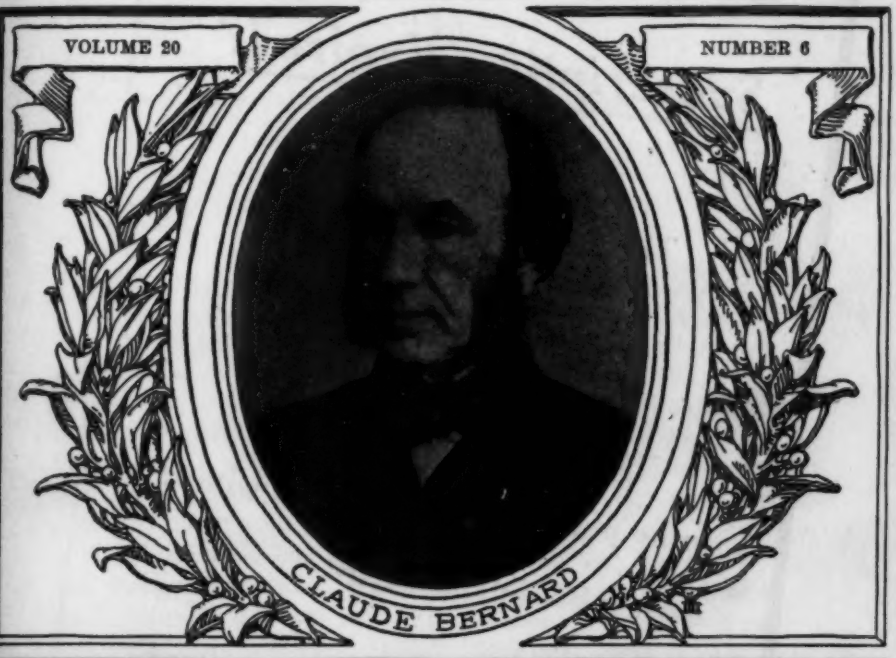

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MULTIPLE DEFICIENCIES IN THE MODIFIED GOLDBERGER DIET AS DEMONSTRATED WITH CHICKS [1]

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TWO FIGURES

(Received for publication July 15, 1940)

In our earlier studies on the chick antidermatitis factor (Mickelsen, Waisman and Elvehjem, '38) numerous attempts were made to produce a nicotinic acid deficiency in chicks through the use of the modified Goldberger diet. We found that chicks on this ration grew very poorly but showed no external symptoms. When the ration was supplemented with various levels of nicotinic acid there was a variable response in growth, indicating that growth was limited mainly by factors other than nicotinic acid.

While our work was in progress, Helmer and Fouts ('38) and Margolis, Margolis and Smith ('39) demonstrated that the Goldberger diet was deficient in thiamin and riboflavin as well as in nicotinic acid. Helmer and Fouts ('38) did their work with rats while the latter workers demonstrated the deficiencies in dogs. Further work in our laboratory showed that the modified Goldberger diet was deficient in several of the factors required by the chick. In this paper we wish to report the results of a rather extensive investigation on this well-known diet using the chick as the experimental animal.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the National Live Stock and Meat Board made through the National Research Council.

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EXPERIMENTAL

The basal ration used in these studies was the modified Goldberger diet which we have fed to dogs in the bio-assay of nicotinic acid (Waisman et al., '40). Its percentage composition is: ground yellow corn 71, purified casein 18, salts 4, cottonseed oil 5, cod liver oil 2. Day-old White Leghorn chicks were used throughout the experiments. They were weighed

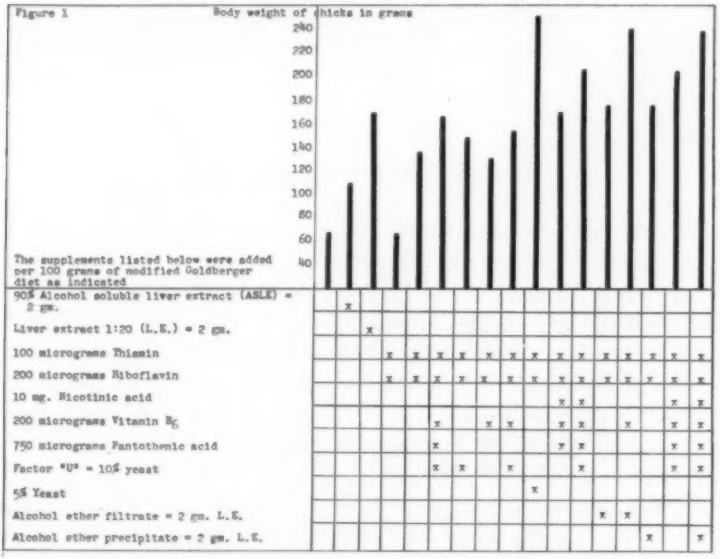

Fig. 1 Average growth responses at 5 weeks of chicks fed various supplements to the modified Goldberger diet.

weekly and carefully observed daily for indications of any symptoms. Water solutions of the supplements were dried on the basal ration. The chicks were given additional vitamins A and D in the form of halibut liver oil twice weekly by dropper.

RESULTS

The results of these experiments are given in very condensed form in figure 1. In many cases several different groups of

chicks were fed the same modifications of the basal ration but we are presenting only the average results. The modified Goldberger ration does not support growth during the assay period of 5 weeks. Mortality is high and the surviving chicks seldom double their original weight in 5 weeks. When the ration was supplemented with 2% of liver extract powder 1:20,[2] growth was fairly good. If the basal ration was supplemented with an alcohol soluble fraction of liver, made from the liver powder after the removal of the pernicious anemia factor, designated in the chart as ASLE, growth was improved over the basal but was far from optimum.

The addition of 100 μg. thiamin per 100 gm. of ration produced only a slight response but a marked growth response was obtained when 200 μg. of riboflavin was added. This result confirms the work of Helmer and Fouts ('38) and Margolis, Margolis and Smith ('39).

Our experiments indicate that some response was obtained in several groups receiving nicotinic acid. Although this ration produces black tongue in dogs and is low in nicotinic acid for this species, the results indicate that this ration does not lend itself for the demonstration that the chick requires nicotinic acid. Although the amounts of nicotinic acid added varied from 10 to 100 mg. per 100 gm. of ration the exact level of the vitamin which gave the best growth response in the chicks could not be determined. In several groups a definite growth gain was noted when the basal diet was supplemented with from 10 to 20 mg. of nicotinic acid per 100 gm. of ration. The growth stimulation by nicotinic acid was also noted when the ration was further supplemented by vitamin B_6, pantothenic acid, and factor U. Our data on a large number of chicks indicate that levels of nicotinic acid above 20–25 mg. per 100 gm. of ration were definitely toxic.

Vitamin B_6 has recently been shown to be essential for the growth of chicks (Carter and O'Brien, '39; Hegsted, Oleson,

[2] Supplies of liver extract were contributed by Wilson Laboratories, thiamin, nicotinic acid, vitamin B_6 and pantothenic acid by Merck and Company, yeast by Pabst Brewing Co., and haliver oil by Abbott Laboratories.

Elvehjem and Hart, '39, '40). When vitamin B_6 was added at a level of 200 μg. per 100 gm. of ration, growth was not improved over the groups receiving thiamin and riboflavin alone. However, when vitamin B_6 was added to the ration containing an alcohol ether filtrate fraction of liver extract (Elvehjem et al., '36), growth was increased above that of the modified Goldberger ration plus the alcohol ether filtrate fraction alone. This is an indication that the growth promoting effect of a particular supplement can be demonstrated only when the ration is more complete in the accessory food factors.

Pantothenic acid has been shown to be the chick antidermatitis factor by Woolley, Waisman and Elvehjem ('39) and by Jukes ('39). We have obtained data indicating that the basal modified Goldberger diet is low in this vitamin. Throughout several groups not receiving supplements of pantothenic acid, a number of chicks showed some dermatitis. Invariably no dermatitis was observed in those chicks which received a pantothenic acid concentrate. A number of groups fed a ration containing factor U (Stokstad and Manning, '38), vitamin B_6, riboflavin, and thiamin showed a definite growth increase when the ration was supplemented with pantothenic acid.

The addition of 5% yeast to the basal ration supplemented with thiamin and riboflavin, resulted in very good growth. The chicks weighed 265 gm. at the end of the 5 weeks' period. This average weight is superior to that of the chicks receiving the five known members of the vitamin B complex, i.e., thiamin, riboflavin, nicotinic acid, pantothenic acid, and vitamin B_6. This increased growth is adequate demonstration that yeast supplies a factor or factors other than the five mentioned above. From this observation we turned to the factor, required by chicks and found in yeast, called factor "U" by Stokstad and Manning ('38). This factor is concentrated by MeOH-H_2O extraction of yeast and subsequent absorption on fuller's earth and elution with NH_4OH or pyridine. This preparation would contain thiamin, riboflavin, vitamin B_6 and certain other factors besides factor U. When such a preparation was fed to the chicks, in amount equivalent to 10% yeast, together with thiamin and riboflavin, a definite response in growth was

obtained. If the factor U concentrate was fed with thiamin, riboflavin, nicotinic acid, vitamin B_6, and pantothenic acid, growth was definitely better than without factor U. However, the average weight of the birds did not reach that of the group on the 5% yeast. Another series indicated that factor U did not give its effect until vitamin B_6 was added. Vitamin B_6 alone did not improve growth nor did the addition of factor U, but when factor U was given together with vitamin B_6 the gain in weight was noteworthy.

Factors which were furnished by the yeast other than factor U may be unidentified members of the vitamin B complex. A definite stimulation to growth was obtained when a liver concentrate, the alcohol ether precipitate (Elvehjem et al., '36), was fed with the basal ration. This stimulation of growth by the alcohol ether precipitate was demonstrated in a number of ways. The response was noted when it was fed either alone or with any combination of the known members of the vitamin B complex. The growth response on alcohol ether precipitate together with any combination of the supplements showed that the factor (or factors) supplied by this concentrate is necessary for optimum growth of the chick fed the Goldberger diet. A definite response above the basal ration was obtained when the alcohol ether filtrate fraction from liver (Elvehjem et al., '36) was fed. This filtrate is known to carry riboflavin, nicotinic acid, and pantothenic acid, besides other factors. The increased growth on this liver fraction is explained by its content not only of known factors, but also by unknown factors, since the growth obtained with the liver fraction was greater than that with the known factors alone.

Interesting results were obtained when a natural foodstuff such as animal tissue was fed with the modified Goldberger ration. Figure 2 shows the growth obtained on various levels of these tissues. In order to conserve space only a few levels of meat are listed. The numbers following the meat samples in figure 2 refer to the sample numbers of the tissues used by us in previous studies. With as little as 1 or 2% pork or lamb liver, or beef kidney, growth was definitely superior to the basal. The growth on the 3% level of beef kidney was equal

to that obtained with 5% yeast. An increasing rate of growth was obtained with the increasing levels of the tissue. With 2 and 4% of veal liver, growth was very good. The addition of 5, 10 and 20% beef spleen to the basal ration resulted in progressively increased growth with increasing quantities of spleen. In one series the birds on the 10% spleen showed definite symptoms (curled toes) of riboflavin deficiency. According to the actual quantity of riboflavin supplied by this tissue (Mickelsen, Waisman and Elvehjem, '39) the riboflavin intake was borderline for the chick (Bethke et al., '37; Norris

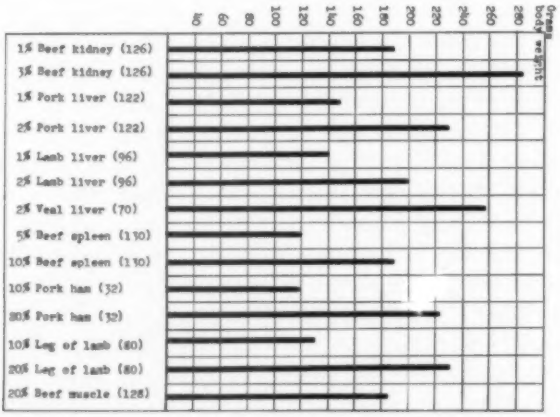

Fig. 2 Growth at 5 weeks of chicks fed various levels of animal tissues together with the modified Goldberger diet.

et al., '36). With the 20% level no symptoms of riboflavin deficiency were observed.

When pork ham was fed at 5 and 10% levels, sufficient thiamin was supplied, but the levels of riboflavin and pantothenic acid were too low to supply the requirements of the chick. It was to be expected therefore that the birds showed definite riboflavin symptoms as well as some typical dermatitis of the pantothenic acid deficiency. Another sample of pork ham was fed at 20 and 30% but the chick showed dermatitis especially at the lower levels. Veal muscle fed at 20 and 30% levels gave

complete protection against any symptoms. With 20 and 30% of beef muscle, growth was good.

The ability of organ tissue to supply most members of the vitamin B complex is greater than that of muscular tissue. This has been a consistent finding in previous studies and is again borne out by the experiments which serve to determine quantitatively the distribution of the members of the vitamin B complex in a particular foodstuff.

The growth studies on the modified Goldberger diet were augmented by observations of the blood picture of the chicks.[3] Hemoglobin, sedimentation rate, red cell count, and hematocrit were determined on most of the groups. We observed a definite lowering of the hemoglobin of the chicks on the basal diet. Hogan and Parrot ('39) have observed an anemia in chicks on a partially synthetic diet. The addition of riboflavin or any liver fraction to the basal diet resulted in increased hemoglobin level accompanied by the increased growth. It appeared that the poor growth was invariably accompanied by low hemoglobin. There was no change in the hematocrit, sedimentation rate, or red cell count when compared to chicks on a normal grain ration. A definite change in plasma color was noted when the group was supplemented with a liver fraction. Further work is being done to establish the cause of this plasma color.

SUMMARY

The limitations of the modified Goldberger ration have been determined with the aid of chicks and supplementation with various vitamins. Thiamin exerts no effect on growth, but riboflavin has been shown to give definite growth responses. Nicotinic acid was found to give variable responses in growth at levels below 20 mg. per 100 gm. of ration. Vitamin B_6 alone did not increase growth over the basal, but when added to a liver fraction which furnished additional factors, a definite response was obtained. The basal ration was found to be low in pantothenic acid and factor U. It has been demonstrated

[3] We are greatly indebted to Dr. Olaf Mickelsen and Mr. LaVell M. Henderson for their aid in some of the observations on the blood work.

that additional factors other than the known members of the vitamin B complex are necessary for optimum growth of chicks fed the modified Goldberger diet. Various levels of animal tissues have been found to give good growth when used as supplements to the basal diet. There is no significant change in the blood picture of the chicks other than a reduced hemoglobin content of the blood when on the basal diet.

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A QUANTITATIVE STUDY OF VITAMINS IN THE RUMEN CONTENTS OF SHEEP AND COWS FED VITAMIN-LOW DIETS

I. RIBOFLAVIN AND VITAMIN K

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FOUR FIGURES

(Received for publication July 27, 1940)

Data regarding the requirements of sheep and cattle for vitamins other than A and D are meager. Theiler, Green and Viljoen ('15) from their results in feeding a vitamin deficient diet to cattle over a period of a year concluded that "it may be that cattle are capable of synthesizing their own vitamins in virtue of the extensive bacterial flora of their intestinal tract." Scheunert and Schieblich ('23) showed that B. vulgatus, an obligate intestinal organism of hebivors is capable of synthesizing the anti-neuritic vitamin. The well-known work of Bechdel and his associates ('26, '27, '28) confirmed the opinion of Theiler, Green and Viljoen ('15) with regard to what was then known as the vitamin B complex. Largely on the basis of this work it has generally been assumed that the whole vitamin B complex may be synthesized in the rumen, and prior to preliminary reports (McElroy and Goss, '39, '40) of the present investigation little qualitative and no quantitative data had been reported in the light of the more recent knowledge of the multiple nature of the B vitamins.

In the Kenya Colony annual report ('34) it is suggested that "sweating sickness" of calves may be due to a deficiency of part of the vitamin B complex in the ration. Madsen, McCay

and Maynard ('35) have reported that sheep and goats maintained on a vitamin B complex deficient diet "require for growth some vitamin B factor found in yeast." Pearson, Schmidt and Mackey ('39) and Winegar, Pearson and Schmidt ('40) have presented evidence indicating that nicotinic acid is not a dietary essential for sheep.

Kramer and co-workers ('39) concluded that the ration of the cow does not have any significant effect on the riboflavin content of the milk.

Little is known regarding the dietary requirement of ruminants for vitamin K. Quick ('37) has shown that the vitamin K in alfalfa is not the factor responsible for its effectiveness in the treatment of sweet clover disease. Phillips and his associates ('38) have suggested that calves maintained on a ration low in vitamin K may suffer from a deficiency of this vitamin.

EXPERIMENTAL

Animals and their care. Experiments of essentially the same nature were conducted with sheep and cows. Four mature grade Dorset Horn ewes from the University flock were housed in a box stall with access to a small corral. No bedding was used and the concrete floor of the stall and corral was kept clean. Throughout most of the experimental period the ewes had a tendency to chew wood but no evidence of coprophagy was noted at any time.

The animals accepted the experimental ration readily and the change from the normal to the experimental ration was completed in 3 weeks. During the final week of the preliminary period only ¼ pound of alfalfa hay per head per day was fed in addition to the experimental ration. No attempt was made to keep individual feed consumption records. The four sheep ate from a common trough and within 30 minutes consumed an allowance of 2100 gm. given twice daily. The average initial weight of 137 pounds per ewe was maintained over the 30-day experimental period.

In the cow experiments three animals were used, but for reasons to be discussed in a later paper most of the vitamin

assays were conducted on rumen material collected from a single cow, a purebred Jersey no. 557, with a permanent fistula [1] into the rumen. The cows were housed in the same quarters as had previously been used for the sheep and were given the same care. The usual feed allowance was 4200 gm. per cow per day.

The fistulated cow no. 557 was maintained on the experimental diet over a period of almost 5 months. After she had been on the experimental ration for 80 days she gave birth to a normal 59 pound heifer calf. From birth to 43 days of age the calf fed solely on her dam's milk and remained in excellent condition gaining an average of 1.7 pounds per day.

Milk production was reasonably good considering the amount of feed the cow consumed (6 kg. per day during lactation). Since the calf was allowed to suckle at will it was not possible to keep accurate milk production records but it was estimated that a peak of between 25 and 30 pounds per day was reached during the second week of lactation. During the third and fourth weeks production fell to an estimated 20 pounds per day and was maintained at this level until the cow was taken off the experimental ration 62 days after parturition. Samples of the milk were saved for use in vitamin assays to be described in this and succeeding papers.

Sheep and cow ration. As previously described (McElroy and Goss, '39, '40) the experimental ration consisted of washed casein [2] 5%, washed sardine meal [3] 6.7, glucose [4] 11.0, corn

[1] For the preparation of the fistula and for assistance in the care of the animal following the operation we are indebted to Dr. G. H. Hart and Dr. H. H. Cole of this Division.

[2] Obtained from the Western Condensing Co., Petaluma, California. In the preparation of washed casein freshly precipitated sulfuric acid casein is washed nine times with five volumes of water brought to pH 4.5 with sulfuric acid. No acid is used in the tenth washing. Washing is effected by stirring the curd with the wash water for 1 hour, allowing it to settle and decanting. The final curd is allowed to drain and then dried in a current of warm air.

[3] Obtained from the Western Condensing Co. Commercial sardine meal is washed ten times with fifteen volumes of hot water (60°–70°C.) and dried in a vacuum oven or in a current of warm air.

[4] "Cerelose."

starch 10.6, mineral mixture 3 and dried plain sugar beet pulp 63.7%. During the later stages of the experiment the cows were given a supplement of 10 cc. per day of a halibut liver oil containing 50,000 U.S.P. units of vitamin A and 850 U.S.P. units of vitamin D per gram. To facilitate mixing of the diet a stock supply of a "concentrate" mixture was prepared. The percentage composition of this mixture was as follows: washed casein 15, washed sardine meal 20, glucose 33, and corn starch 32. The mineral mixture used was a modification of that described by Hubbell, Mendel and Wakeman ('37) and had the following composition (in grams):

$CaCO_3$	1500
KH_2PO_4	3000
NaCl	3000
Ferric citrate	200
$MgCO_3$	200
$CuSO_4 \cdot 5\ H_2O$	7.4
$K_2SO_4Al_2(SO_4)_3$	1.4
$MnSO_4 \cdot 7\ H_2O$	7.0
KI	0.66

The ration was prepared before each feeding by mixing 33.3 parts of the "concentrate" mixture, 3 parts of the mineral mixture and 63.7 parts of dried plain sugar beet pulp. This was moistened with two to three times its weight of water and was fed immediately in a clean trough.

Collection and treatment of rumen contents. The sheep were kept on the experimental ration for 30 days. It was assumed that after this length of time any residue of feed other than the experimental ration would have left the rumen. The sheep were then slaughtered 14 hours after the last feeding and the rumen and reticulum contents of each animal emptied into a separate stone crock. Enough 95% ethanol was added to bring the concentration of alcohol in the mixture to about 50%. This material was then stored in the cold and subsequently dried in glass dishes before a current of air at room temperature. The drying process required from 48 to 72 hours. When dry, the material was finely ground in a Wiley mill and stored in closed containers at room temperature until used in assay

diets. In all assays of sheep rumen and reticulum contents composite samples of equal weights of material from each of the four sheep were used.

The first sample of rumen contents was removed from fistulated cow no. 557 after she had been on the experimental diet for 43 days. During the following 3½ months eleven additional samples of between 25 and 35 pounds wet weight were taken at intervals varying from 4 to 16 days. Samples were siphoned out through a ¾ inch rubber tube into stone jars. The rumen material was relatively liquid so that little difficulty was experienced in maintaining the siphon, and since care was taken to move the tube to different regions and levels of the rumen during the process of drawing the sample it is believed that this method permitted the collection of a representative sample of the rumen contents.

The sheep rumen and reticulum contents described above were taken in the summer when the atmospheric temperature was high and the humidity low so that rapid drying was obtained at room temperature, but due to the lower temperature and higher humidity prevailing while the cow rumen samples were being taken this method of drying could not be used for the cow samples. They were therefore dried before a current of air in an electrically heated oven at a temperature of 40° to 50°C. The first two samples were dried immediately after the addition of enough 95% ethanol to make the concentration of alcohol approximately 30%. The succeeding ten samples were first treated with alcohol and then with enough 36% HCl to lower the pH to 4.5 to 5.0 before being dried. In a chick assay described below it was found that a composite mixture of samples 3 and 4 which were acidified before drying contained about twice as much riboflavin as a composite mixture of samples 1 and 2 which were dried without the addition of acid. In the light of this finding all but one of the subsequent assays of cow rumen contents for riboflavin, as well as the other vitamins under study were made on material which had been treated with both alcohol and acid as soon as it was removed from the rumen.

Riboflavin assays of sheep rumen and reticulum contents. For the riboflavin assays a modification of the method of Jukes ('37) was used. In this method the growth rate of chicks receiving basal diet 99 (Jukes, unpublished) deficient in riboflavin, supplemented with the test material, is compared with the growth rate of groups of chicks receiving the basal diet supplemented with various levels of synthetic riboflavin.[5]

Diet 99 had the following composition:

Ground barley	30 grams
Glucose [6]	37
Washed sardine meal [7]	23
Liver filtrate [8]	6 cc.
Ground limestone	1 gram
Iodized salt	0.45
$MnSO_4$	0.05
Soybean oil	3
Fish oil blend, 3000 U.S.P. units of vitamin A and 400 A.O.A.C. units of vitamin D per gram	0.3
Thiamine chloride hydrochloride	100 μg. per 100 gm. of diet

Liver filtrate used in diet 99 was freed of riboflavin as follows: 1 kg. of liver extract powder (Lilly 343) was dissolved in 5 to 6 liters of warm water and 1 liter of 95% ethanol was added to inhibit bacterial action. One kilogram of English fuller's earth was added and the mixture was stirred for 30 minutes. The fuller's earth was removed by filtration and washed with 500 cc. of warm water. The fuller's earth was discarded and the combined filtrate and washings were again treated with 1 kg. of fresh fuller's earth. The process was repeated once more and the combined filtrate and washings were concentrated under reduced pressure to a volume of 1400 cc. Unpublished experiments conducted in the Poultry Division of the University of California, Davis, have shown that such preparations when fed at a level of 6% in diet 99,

[5] Merck and Company.

[6] See footnote 4, page 529.

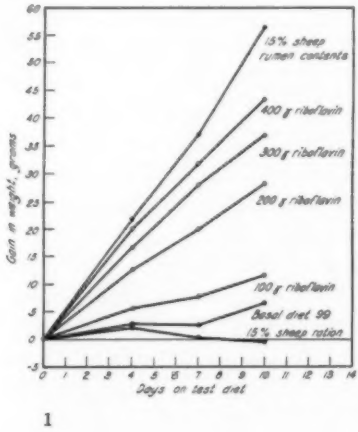
[7] See footnote 3, page 529.

[8] Ely Lilly and Company, Indianapolis, kindly supplied the liver extract powder (Lilly 343) from which the filtrate was prepared.

supplemented with riboflavin, supply sufficient pantothenic acid and possibly other as yet unidentified water-soluble factors for normal growth of the chick.

RESULTS

In the first assay the dried sheep rumen and reticulum contents were fed at a level of 15% and in the second at a level of 5%. The results obtained are summarized in figures 1 and 2. Figure 1 shows that in the first trial the sheep rumen and reticulum contents were fed at a level too high to furnish a satisfactory assay, the growth made by this group exceeding



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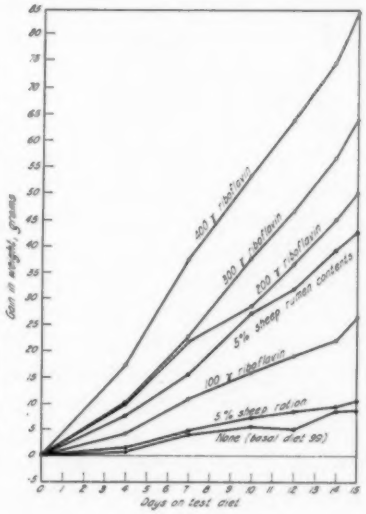

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Fig. 1 First riboflavin assay of sheep ration and of dried rumen and reticulum contents. The chicks were fed a normal diet for the first 7 days, then depleted on basal diet 99 for 8 days following which they were divided into experimental groups of ten chicks each. Supplements added to basal diet 99 are indicated opposite the curves. The amounts of riboflavin shown were added per 100 gm. of diet.

Fig. 2 Second riboflavin assay of sheep ration and of dried rumen and reticulum contents. The number of chicks used in each group and their previous treatment was the same as for figure 1 except that the birds were depleted for 7 rather than 8 days.

that made by any of the positive control groups. No riboflavin was detected in the sheep ration. Since Jukes (unpublished data) has estimated that diet 99 contains no more than 0.3 μg. of riboflavin per gram these results indicate that the experimental sheep and cow ration contained less than 0.3 μg. of riboflavin per gram.

Figure 2 shows that the growth curve for the group fed 5% sheep rumen and reticulum contents was equivalent to that which might be expected with birds receiving 165 μg. of riboflavin per 100 gm. of diet. The riboflavin content of the dried sheep rumen and reticulum contents was therefore estimated as 33 μg. per gram. The curves for the basal and 5% sheep ration groups again show that no riboflavin was detected in the sheep ration.

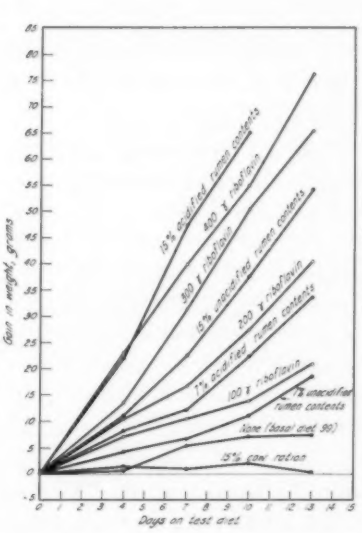
Riboflavin assays of cow ration, rumen contents and dried skim milk from fistulated cow no. 557. Two riboflavin assays of dried rumen contents and one of dried skim milk from cow no. 557 were completed. The method of assay was the same as described above for sheep rumen and reticulum contents. One change was made in diet 99, namely, the replacement of the liver filtrate with 7% rice bran filtrate.[9]

As previously mentioned, the first two samples of rumen contents taken from cow no. 557 were dried immediately after the addition of enough 95% ethanol to make the concentration of alcohol approximately 30%, while the succeeding samples were treated with alcohol and then with enough concentrated HCl to lower the pH to 4.5–5.0 before being dried. The reason for the addition of acid before drying is apparent from figure 3 which summarizes the results of the first riboflavin assay of cow rumen contents. In the 13-day assay period the group receiving 7% acidified rumen contents gained 26.2 gm. more per bird than the basal group as compared with a net gain of 11.2 gm. for the birds receiving 7% unacidified supplement.

[9] The rice bran filtrate used as a source of pantothenic acid was the "Chicken Riboflavin Assay Supplement" sold by the Vitab Corporation, Emeryville, California. Riboflavin is removed from the product by repeated treatments with fuller's earth.

At the higher level of 15% the difference between the effectiveness of the acidified and unacidified supplement was also marked.

These results indicate that drying the rumen contents at the natural pH (6.8–7.8) caused a distinct loss of riboflavin potency. No comparative assays were run to determine if a similar loss occurred with any of the other vitamins under study. As previously mentioned the sheep rumen and reticulum contents, found by biological assay to contain 33 μg. of



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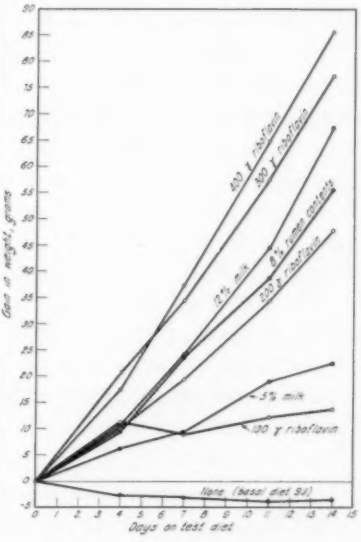

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Fig. 3 First riboflavin assay of cow ration and of dried cow rumen contents. The chicks were fed a normal diet for 8 days after hatching and were then depleted on basal diet 99 for 7 days. They were then divided into test groups of ten chicks each. Supplements added to basal diet 99 are indicated above the curves. The amounts of riboflavin shown were added per 100 gm. of diet.

Fig. 4 Riboflavin assay of dried rumen contents and of dried skim milk from cow no. 557. The chicks were fed a normal diet for 7 days and were then depleted on diet 99 for 8 days. They were then divided into experimental groups of ten chicks each. Supplements added to basal diet 99 are indicated above the curves. The amounts of riboflavin shown were added per 100 gm. of diet.

riboflavin per gram, were dried at room temperature without the addition of acid. The loss in riboflavin potency during drying may therefore have been appreciable but in view of the lower temperature used (not exceeding 30°C.) this loss would presumably be less than that found for cow rumen contents dried at 40°–50°C.

From the growth curves shown in figure 3 it was estimated that the inclusion of 7% acidified cow rumen contents in diet 99 provided at least 150 μg. of riboflavin per 100 gm. of diet. The riboflavin potency of dried cow rumen contents would therefore be at least 21 μg. per gram.

The growth curve for the group receiving 15% cow ration shows that no riboflavin was detected in the ration. Similar results are shown in figures 1 and 2.

In the second riboflavin assay of no. 557 cow rumen contents a composite mixture of equal weights of three samples taken 57, 66 and 91 days after the cow had been placed on the deficient ration was used in making up the test diets. These samples had all been acidified before drying and the mixture was fed at a level of 8%. As shown in figure 4 the group receiving this supplement made greater growth than the group supplemented with 200 μg. of synthetic riboflavin per 100 gm. of diet. From these results the average riboflavin content of the three rumen contents samples was estimated to be at least 25 μg. per gram.

In this same assay dried skim milk from cow no. 557 was tested at levels of 5% and 12%. The skim milk was prepared by concentrating under reduced pressure and evaporating to dryness in a current of air at 40°C. Equal weights of two dried skim milk preparations were used in making up the supplement to diet 99. The milk used for these preparations was collected between the seventeenth and twenty-fourth and between the forty-fifth and forty-ninth days of lactation. The growth response (fig. 4) in the groups receiving supplements of dried skim milk indicated that the riboflavin content of the skim milk was at least 20 μg. per gram on a dry basis.

In preliminary experiments the fluorescence test of Whitnah et al. ('37) was used to compare whole milk from cow no. 557 with whole milk from cows on a normal winter ration in the University herd. As reported elsewhere (McElroy and Goss, '40) the milk from the experimental cow was found to contain 1.4 ($\pm$0.15) μg. of riboflavin per gram as compared with an average of 1.5 ($\pm$0.25) μg. for the milk from twelve Jersey cows in the normal herd.

The conclusion that this cow, fed a ration practically free of riboflavin, produced milk of normal riboflavin content is further substantiated by the fact that the riboflavin values obtained by biological and fluorescence measurements are

TABLE 1

Vitamin K assay of cow ration and of dried cow rumen contents

SUPPLEMENT TO K-FREE DIET	LEVEL	NO. CHICKS	AVERAGE PROTHROMBIN TIME, SECONDS
	%		
Cow basal diet	10	6	166
Cow rumen contents	5	7	28.5
Cow rumen contents	15	7	23.8
Reference standard alfalfa	0.8	6	28.1
Practical chick mash	100	3	28.3

within the range reported for milk from cows fed a normal ration by Whitnah et al. ('37, '38), Jukes and Richardson ('38), Hodson and Norris ('39) and Hand ('39).

Vitamin K assay of no. 557 cow rumen contents and cow ration. This assay was undertaken at the suggestion of Dr. H. J. Almquist, Poultry Division, University of California, Berkeley, and we wish to acknowledge our appreciation to Doctor Almquist for having conducted the assay here reported.

The experimental ration and a composite mixture of rumen contents collected from cow no. 557, 57, 66 and 91 days after she had been placed on the diet were assayed for vitamin K by the method of Almquist and Klose ('39). The results are summarized in table 1.

These data show that the dried rumen contents of the cow was a good source of vitamin K, being about one-sixth as potent as the standard dried alfalfa. That the cow ration was practically free of the vitamin is indicated by the fact that the prothrombin time for the group supplemented with 10% of the cow ration was typical of the usual results obtained on the vitamin K-free diet alone.

Phillips, Rupel, Oleson and Bohstedt ('38) have reported improved blood-clotting times for calves when a diet apparently deficient in vitamin K was supplemented with cottonseed oil [10] and have suggested that this improvement was due to vitamin K supplied by the oil. The results of the prothrombin time assay reported in this paper indicate that under the conditions of our investigation a relatively large amount of vitamin K was synthesized in the rumen, and it would seem that the beneficial effect of cottonseed oil on blood-clotting time reported by Phillips et al. may have been due to some factor other than vitamin K.

SUMMARY

The rumen contents of four sheep fed a ration containing less than 0.3 μg. of riboflavin per gram were found to contain 33 μg. of riboflavin per gram.

Evidence for the formation of riboflavin in the digestive tract of a cow fed the same deficient ration has been presented in two ways.

1. The dried rumen contents of this animal contained approximately 25 μg. of riboflavin per gram.

2. The skim milk contained at least 20 μg. of riboflavin per gram on a dry basis, or 1.8 μg. per cubic centimeter on a fluid basis using a figure of 9% for solids in skim milk. The average daily milk production was between 9 and 10 liters and the secretion of riboflavin in the milk was therefore estimated to be between 16 and 18 mg. per day as compared with a maximum intake of 1.8 mg. per day in the feed.

[10] Wesson.

That vitamin K is not a dietary essential for the cow is indicated by the finding that, although the experimental ration was practically free from this vitamin, the rumen contents of the experimental animal proved to be a good source of the factor.

ACKNOWLEDGMENT

It is a pleasure to acknowledge the many helpful suggestions of Dr. T. H. Jukes. We are indebted to him also for his generous provision of facilities in his laboratory for conducting the riboflavin assays.

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A QUANTITATIVE STUDY OF VITAMINS IN THE RUMEN CONTENTS OF SHEEP AND COWS FED VITAMIN-LOW DIETS

II. VITAMIN B_6 (PYRIDOXINE)

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TWO FIGURES

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We have briefly reported (McElroy and Goss, '39) that the dried mixed rumen and reticulum contents of sheep, fed a ration deficient in the vitamin B complex was a good source of vitamin B_6. The investigation has now been extended to include assays for vitamin B_6 in dried rumen contents and skim milk from a cow fed the deficient ration. The complete results of the study of vitamin B_6 synthesis in these two species are reported in the present paper.

EXPERIMENTAL

The animals used, their care and ration, and the method of collection and treatment of the rumen contents have already been described (McElroy and Goss, '40 a).

Method of assay for vitamin B_6. The method used in assays for vitamin B_6 was a modification of that described by Dimick and Schreffler ('39). The diet of the positive control rats was supplemented with synthetic vitamin B_6 hydrochloride[1] instead of the crystalline factor I of Lepkovsky ('38). For the assay of sheep rumen and reticulum contents a basal rat diet

[1] Kindly furnished by Merck and Company, Rahway, New Jersey, through the kindness of Dr. D. F. Robertson.

with the following composition was used: glucose [2] 60 parts, washed casein (McElroy and Goss, '40 a) 27, hydrogenated cottonseed oil [3] 8, salt mixture (Jukes, '39), 5, and fish oil blend containing 3000 U.S.P. units of vitamin A and 400 A.O.A.C. units of vitamin D per gram, 0.2 parts.

In the assay of cow rumen contents and milk from the experimental cow this basal rat diet was changed to include 4 parts hydrogenated cottonseed oil and 4 parts lard. This change was made to avoid the possibility of a deficiency of unsaturated fatty acids in the basal diet. Since no difference was noted in the response of the rats in the two assays it is believed that the first basal ration, used for the assay of vitamin B_6 in sheep rumen material, was satisfactory even though no precaution was taken to provide additional unsaturated fat.

The test animals used were female rats of the Long-Evans strain, weaned at 21 days and placed in groups of three to four in cages with raised wire bottoms. For the first 2 weeks after weaning they were fed only the basal diet. From the third week on each individual was fed 0.2 cc. of a supplement containing 15 μg. of thiamine chloride hydrochloride and 30 μg. of riboflavin dissolved in a 10% solution of ethanol. Factor II was supplied from the beginning of the fifth week to the end of the assay. In the assay of sheep material a liver filtrate [4] was used for this purpose which had been prepared by five treatments with fuller's earth after the method of Lepkovsky, Jukes and Krause ('36). After concentration under reduced pressure the filtrate was treated with 8 volumes of 95% ethanol. The precipitate brought down by this treatment was discarded and the final filtrate was again concentrated so that 1 cc. of filtrate represented 2 gm. of the original liver extract powder. No tests were run to determine the potency of this preparation but it is believed that the dose fed (0.5 cc. daily) provided ample factor II for normal growth of the rat.

[2] Cerelose.

[3] Crisco.

[4] The liver extract powder (Lilly 343) from which the filtrate was prepared was kindly furnished by Ely Lilly and Company, Indianapolis.

In the assay of cow material the liver filtrate used was prepared from liver extract 70 A.S.[5] by similar treatment with fuller's earth and alcohol. This preparation was fed at a level of 0.2 cc. daily since preliminary experiments showed that this amount supplied sufficient factor II to satisfy the growth requirement of the rat.

The rats were maintained on the depletion diet until growth had practically ceased and dermatitis had developed. In addition to the usual dermatitis of the face, ears and paws a "ring-tailed" condition (McElroy and Goss, '40 b) developed in a number of the test animals.

At the end of the depletion period of about 7 weeks the rats were divided into uniform test groups. All groups continued to receive the previous supplements of thiamine, riboflavin and liver filtrate preparation, while the two positive control groups received a further daily supplement of 0.2 cc. of an aqueous solution containing 3 and 10 μg. respectively of synthetic vitamin B_6 hydrochloride.

Results of assay of sheep ration and of sheep rumen and reticulum contents. The growth made by the different groups in this assay is shown in figure 1. A comparison of the growth curves for groups V and VI indicates that the sheep ration probably contained a small amount of vitamin B_6. There was some evidence of healing of the dermatitis in three members of the group fed the basal diet supplemented with 20% sheep ration; of the other four, three remained unchanged and one developed a more severe dermatitis.

The growth curves for groups I and III show that the rumen and reticulum contents contained much more vitamin B_6 than did the sheep ration. Group I, whose diet was supplemented with 20% dried sheep rumen and reticulum contents, gained an average of 60 gm. per rat in 13 days, and exhibited rapid and uniform healing of the dermatitis throughout the lot. Feed consumption in this group was very high, and since only a limited amount of the rumen material was available these

[5] Kindly furnished by the Armour Laboratories, Chicago, Illinois, through the courtesy of Dr. E. F. Pike.

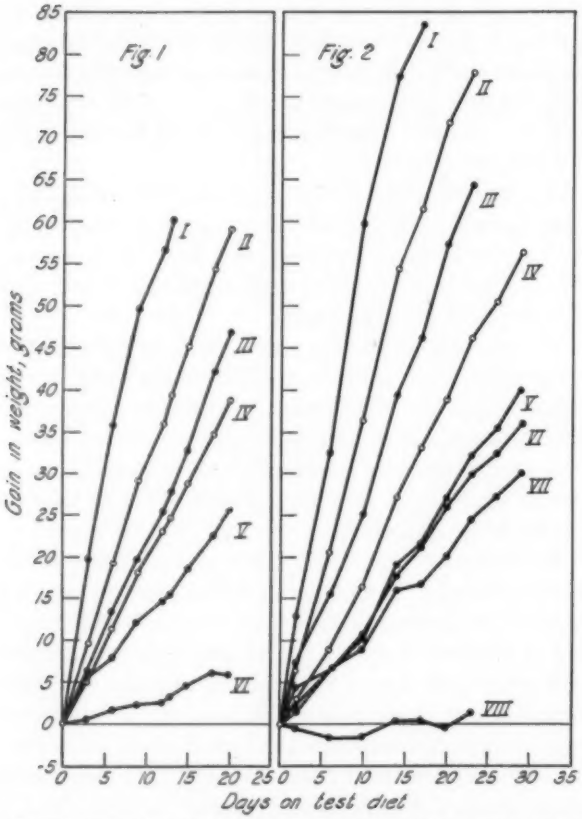

Fig. 1 Growth curves for rats in vitamin B_s assay of sheep ration and of dried sheep rumen and reticulum contents. Seven female rats were included in each group. The basal diets of the groups were supplemented as follows: I. 20% dried sheep rumen and reticulum contents. II. 10 μg. vitamin B_s daily. III. 5% dried sheep rumen and reticulum contents. IV. 3 μg. vitamin B_s daily. V. 20% sheep ration. VI. None (basal only).

Fig. 2 Vitamin B_s assay of cow ration and of dried rumen contents and skim milk from cow no. 557. Six female rats were included in groups I, III, IV and VIII, and five in groups II, V, VI and VII. The basal diets of the groups were supplemented as follows: I. 15% dried rumen contents. II. 10 μg. vitamin B_s daily. III. 5% dried rumen contents. IV. 3 μg. vitamin B_s daily. V. 4 cc. concentrated skim milk daily. VI. 15% cow ration. VII. 2 cc. concentrated skim milk daily. VIII. None (basal only).

rats were killed on the thirteenth day of the assay. Group III, fed 95 gm. of the basal diet plus 5 gm. of dried rumen and reticulum contents gained an average of 46.7 gm. per rat over the 20-day assay period as compared with 38.7 and 58.9 gm. for the groups receiving a daily supplement of 3 and 10 μg. of synthetic vitamin B_6 hydrochloride. The degree of healing of the dermatitis in the group fed 5% rumen and reticulum contents was intermediate between that observed in the two positive control groups.

Group III, fed 5% dried sheep rumen and reticulum contents, ate an average of 9.8 gm. of feed, or the equivalent of approximately 0.5 gm. of test substance per rat daily. Since the consumption of this amount of rumen and reticulum material caused growth and healing which might be expected with rats receiving 5 μg. of vitamin B_6 daily, it was estimated that the dried rumen and reticulum contents contained approximately 10 μg. of vitamin B_6 per gram.

Results of assay of cow ration and of dried rumen contents and skim milk from fistulated cow no. 557. The dried rumen contents of fistulated cow no. 557 was tested at 5% and 15% levels. Aliquot portions of rumen material removed 57, 66 and 91 days after the cow had been placed on the deficient ration were used in making up the test diets. In testing the milk a composite sample collected during the forty-fifth to the forty-ninth days of lactation was used. This sample had previously been skimmed in an ordinary cream separator and then concentrated under reduced pressure to one-third of its initial volume. The test groups received 2 cc. and 4 cc. per rat daily of this preparation or the equivalent of 6 cc. and 12 cc. of skim milk.

The results of the assay are summarized in figure 2. The growth response in group IV supplemented with 3 μg. of vitamin B_6 per day was practically identical with that of the corresponding group IV in figure 1. The degree of healing of dermatitis obtained with this amount of the pure vitamin was also duplicated. When 10 μg. of vitamin B_6 was fed, healing

progressed at the same rate in both assays but growth was somewhat more rapid in the second one.

Group VI (fig. 2), whose diet was supplemented with 15% cow ration, made almost the same growth as the group fed 20% of the same ration in the assay conducted on sheep material (fig. 1). In both assays the sheep or cow ration groups failed to make as much growth as the lowest positive control group (3 μg. vitamin B_6 per day) and only a rough estimate may be made for the vitamin B_6 content of the ration fed to the experimental sheep and cows. By assuming that the growth made was equivalent to that which would be made by rats fed 2 μg. of vitamin B_6 per day, and calculating from feed consumption records the amount of sheep (or cow) ration consumed, it was estimated that the experimental sheep and cow ration contained between 1 and 1.5 μg. of vitamin B_6 per gram.

From figure 2, groups II, III and IV, it was estimated that the rats in group III obtained at least 5 μg. of vitamin B_6 per day. The average feed consumption in this group was 12.0 gm. or 0.6 gm. of rumen contents per rat daily. The vitamin B_6 content of the dried cow rumen material was thus estimated to be at least 8 μg. per gram, which is about the same potency as estimated for dried sheep rumen and reticulum contents.

As shown in figure 2, the group fed 15% cow rumen contents gained weight more rapidly than the group whose diet was supplemented with 10 μg. of vitamin B_6. The appetite of these rats improved immediately when they were placed on the test diet, as is indicated by the fact that they gained an average of 13 gm. per rat during the first 2 days of the assay. Their average daily feed consumption for the assay period was 16 gm. per rat. The rats of this group were indistinguishable from normal animals when they were removed from the experiment on the seventeenth day. It was observed that, in all groups supplemented with either sheep or cow rumen material, food wastage became negligible as soon as the supplement was added.

The growth curve and healing response of group V, which received 4 cc. of concentrated skim milk per rat daily, indicated that these animals were receiving about 2 μg. of vitamin B_6 per day. Since the milk was concentrated 3:1, the vitamin B_6 content of the original skim milk was estimated as 0.17 μg. per cubic centimeter. No recent vitamin B_6 assays of cow's milk have come to our attention, but György ('36) has reported vitamin B_6 assays of cow's milk in which a daily dose of 15 cc. of milk resulted in an average weekly increase of 14 gm. per rat. The corresponding figure from the present assay was 10 gm. per rat with a dose equivalent to 12 cc. of skim milk. Since György provided only vitamin B_1 and riboflavin in his test

TABLE 1

A comparison of the growth of rats depleted of vitamin B_6 for 71 days and then fed for 21 days the depletion diet plus 20% of either commercial skim milk powder or skim milk powder made from the milk of cow no. 557

SUPPLEMENT	RAT NO.	WEIGHT IN GRAMS AT 3-DAY INTERVALS								GAIN IN GRAMS	
		Initial	3	6	9	12	15	18	21	Total	Avge. daily
Commercial	1	91	97	103	110	112	117	121	123	32	1.5
skim milk	2	87	88	93	99	104	110	119	127	40	1.9
powder	3	54	52	Dead 5th day							
Cow no. 557	4	68	71	79	92	97	100	109	117	49	2.3
skim milk	5	90	105	107	110	118	123	128	140	50	2.4
powder	6	124	125	135	140	148	151	158	168	44	2.1

diet, his results are not strictly comparable with those of the present investigation, in which the test diet was supplemented with "factor II" (Lepkovsky, Jukes and Krause, '36) in addition to thiamine and riboflavin. However, a comparison of the results obtained at least suggests that the experimental cow, fed a ration deficient in vitamin B_6, produced milk containing a normal amount of this vitamin.

A direct comparison was made of the vitamin B_6 content of commercial skim milk powder prepared by the drum process and that of skim milk powder prepared from milk collected from our experimental cow between the seventeenth to twenty-sixth and the forty-fifth to forty-ninth days of lacta-

tion. This skim milk powder was prepared by first concentrating under reduced pressure and then drying to a constant weight before a current of air at approximately 40°C.

The six rats (group VIII, fig. 2) included in the basal group of the vitamin B_6 assay just described were used for this assay. These animals had been on the depletion diet for 71 days and were consequently in very poor condition. Two of them, nos. 3 and 4, were not expected to recover when their diet was changed to include 20% skim milk powder. As shown in table 1, rat no. 3 died 5 days after the diet was changed but no. 4 recovered and made as rapid growth as any of the other animals. Although the number of rats included is small, table 1 shows that the skim milk powder prepared from the milk of cow no. 557, fed a ration deficient in vitamin B_6, contained fully as much of this vitamin as did the commercial product.

DISCUSSION

The ratio of vitamin B_6 potency of rumen contents to the potency of the ration was found to be between 6 and 8 to 1. The question arises whether this represents merely a concentration of the vitamin due to differential rates of passage of different nutrients from the rumen, or whether there has been a true synthesis related to the growth of the rumen flora. In our opinion there are reasons why the latter view should be favored. The amount of vitamin B_6 present in the milk of cow no. 557 was apparently normal which indicates that the cow received enough vitamin to meet her maintenance metabolic needs and was able in addition to transfer the normal amount through the mammary gland into the milk. The chief weakness of this argument resides, of course, in our lack of knowledge of the actual requirement of the cow for vitamin B_6.

Such data as were obtained concerning the differential rates of passage of materials from the rumen are open to different interpretations. For example, the ration contained 0.42% fat

and the rumen contents of the fistulated cow 2.56%. The corresponding figures for protein were 15.2% and 29.8%. On the other hand, the nitrogen-free extract values were 59.0% and 17.8% respectively. In view of its solubility it might be argued that vitamin B_6 would be more comparable to the nitrogen-free extract material than to the protein or fat in its rate of passage from the rumen, and from this point of view, one would be led to conclude that the vitamin had been formed in the rumen.

SUMMARY

The dried rumen and reticulum contents of four sheep fed a ration containing between 1 and 1.5 μg. of vitamin B_6 per gram were found to contain approximately 10 μg. of vitamin B_6 per gram.

The dried rumen contents of a fistulated cow fed the same ration was estimated to contain 8 μg. of vitamin B_6 per gram.

Milk from the cow fed the deficient ration contained at least as much vitamin B_6 as milk from cows fed a normal ration.

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STUDIES ON THE METABOLISM OF NICOTINIC ACID IN THE SHEEP [1]

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TWO FIGURES

While the significance of nicotinic acid in the nutrition of man, dogs and pigs is well established, information on its dietary importance for ruminants is limited. On the basis of growth studies (Pearson, Schmidt and Mackey, '39) evidence was presented indicating that nicotinic acid is not a dietary essential for sheep. McElroy and Goss ('40) reported an increase in the nicotinic acid of the rumen contents of a cow fed a diet deficient in this constituent. Winegar, Pearson and Schmidt ('40) reported that sheep restricted to a diet low in nicotinic acid continued to excrete substantial amounts in the urine. Thus it appears that nicotinic acid is formed in the body of sheep, since animals for which it is a dietary essential virtually cease to excrete it when restricted to a deficient diet. We have continued our investigations on the metabolism of nicotinic acid in ruminants and have studied the effect of the intake on the amounts in the blood and urine, the rate of elimination and recovery of massive doses.

EXPERIMENTAL

Seven lambs approximately 10 weeks of age and weighing between 9 and 12 kg. were placed on a diet having a percentage

[1] Published with the approval of the Director of the Texas Agricultural Experiment Station as technical contribution no. 615.

composition of regenerated cellulose 20, brewers' rice 50.5, purified casein [2] 9, yellow corn 16, cow peas 2.5 and salts mixture (Hughes, '38) 2. Vitamins A and D in addition to that furnished through natural sources were supplied by oleum percomorphum or other concentrated fish oils at weekly intervals. Originally riboflavin was included in the diet but was later omitted when it was reported (McElroy and Goss, '39) that this vitamin is synthesized in the rumen of sheep.

The nicotinic acid content of the various components of the diet was determined photometrically by the technique of Bandier ('39 a) with the modification that aniline was used in place of metol. On the basis of the chemical assay the diet contained 0.6 mg. of nicotinic acid per 100 gm.

The diet was also assayed with dogs for its blacktongue producing properties. In order to insure an adequate caloric intake for the dogs the regenerated cellulose was omitted. The cellulose was included in the diet for the sheep merely to provide the necessary bulk. This alteration in the diet has no material bearing on the nutritional value as lignified cellulose is almost entirely undigestible even by ruminants (Crampton and Maynard, '38). Dogs fed this diet showed a marked decrease in their urinary nicotinic acid and developed typical blacktongue symptoms.

Growth response. Early in the experiment two lambs died from undeterminable causes leaving only five on which complete data are available. The growth response of all of the lambs was very unsatisfactory as there was only a slight increase in weight during an experimental period of 214 days. This was probably due to the unpalatability of the diet and the resulting low feed consumption which averaged only 408 gm. daily as compared with 934 gm. for lambs receiving a similar diet except that corn was used in place of brewers' rice (Pearson et al., '39). During the last 160 days lamb no. 447 was fed 10 gm. of dried brewers' yeast per day. Since the yeast failed to bring about any response in either appetite or growth further support is afforded the belief that the poor growth

[2] Glaxo.

performance was not due to a deficiency of members of the vitamin B complex. The unfavorable growth response does not affect the validity and interpretation of the chemical data as will be shown later by comparison with results obtained with lambs on a standard stock diet.

Blood studies. At monthly intervals the nicotinic acid content of the blood was determined photometrically by the procedure of Pearson ('39). This method with the slight modification that the nicotinic acid is adsorbed on fuller's earth has been used successfully with blood by Villela ('40).

TABLE 1

Nicotinic acid content of blood

LAMB NUMBER	DIET	RANGE	AVERAGE
		mg./100 ml.	*mg./100 ml.*
418	Deficient	0.48–1.52	0.93
420	Deficient	0.63–1.18	0.87
443	Deficient	0.65–1.70	1.00
488	Deficient	0.59–1.14	0.84
447	Deficient	0.72–1.27	1.01
447	Deficient + yeast	0.60–1.27	0.92
523	Stock [1]		0.61
537	Stock [1]		1.54
550	Stock [1]		0.81
558	Stock [1]		0.62
3445	Stock [1]		0.97

[1] One analysis.

While there was considerable variation in the values for individual lambs during the course of the experiment, the magnitude was about the same as that previously reported (Pearson, '39) for normal lambs on a similar diet. There was no consistent trend in the nicotinic acid content of the blood as the experiment progressed. For this reason only the range and average values for the 7-month period are presented in table 1. There was no consistent increase in the nicotinic acid level of the blood of lamb no. 447 after yeast was included in the diet. Furthermore, there is no essential difference between the values of the lambs on the deficient diet and those

receiving a stock diet consisting of corn, alfalfa hay, cottonseed meal, and a limited amount of green grass. These observations are of interest in connection with those of Ballif et al. ('39) who found no reduction in the nicotinic acid amide content of the blood of ten pellagrins. Likewise there appears to be no decrease in the coenzyme content of the blood of pellagrins (Kohn, '38), dogs and pigs fed deficient diets (Kohn, Klein and Dann, '39; Axelrod, Madden and Elvehjem, '39). On the other hand it has been shown that the coenzyme content of the blood can be increased by feeding nicotinic acid (Kohn, '38; Axelrod, Gordon and Elvehjem, '40). The latter workers fed as much as 1.8 gm. in a period of 16 hours. The ingestion of this relatively large amount as compared with the intake of the lambs on the stock diet may be a factor in accounting, at least in part, for the increase in the coenzyme content of the blood of pellagrins and the failure to observe a difference in the nicotinic acid level of the blood of lambs on the deficient diet and those receiving a stock diet or a supplement of yeast. In fact it will be shown later that the ingestion of large doses of nicotinic acid increases by severalfold the level in the blood. A second factor, as will be pointed out later, is that nicotinic acid is synthesized in the body of this species; thus adequate amounts are available to maintain normal levels in the blood.

Urinary nicotinic acid. The determination of the nicotinic acid of the urine of sheep on a deficient diet (Winegar, Pearson and Schmidt, '40) was made on the unhydrolyzed urine by the aniline and cyanogen bromide reaction as described by Pearson and Winegar ('40). Since this work was done there have appeared several papers (Bandier, '39 b; Harris and Raymond, '39; Swaminathan, '39) showing that a part of the nicotinic acid may be excreted as nicotinamide, nicotinuric acid and the codehydrogenases. These conjugated forms can be converted to the free nicotinic acid by alkaline hydrolysis. It was, therefore, of interest to study the effect of alkaline hydrolysis on the nicotinic acid values of the urine of normal

sheep with a view to the possible reinterpretation of the data obtained from the sheep on the deficient diet.

The urine was hydrolyzed and the nicotinic acid determined by the method of Harris and Raymond ('39) with the modification that aniline was used in place of para-aminoacetophenone. The urine was collected quantitatively over a 24-hour period from lambs weighing between 17 and 23 kg. The figures in table 2 are average values for 3 consecutive days. Subjecting the urine to alkaline hydrolysis for 30 minutes resulted in increases of from 12.6 to 31.6% in the nicotinic acid values.

TABLE 2

Nicotinic acid values in urine before and after hydrolysis

LAMB NUMBER	UNHYDROLYZED	HYDROLYZED	INCREASE FROM HYDROLYSIS
	mg./24 hr.	*mg./24 hr.*	%
523	3.27	4.10	25.4
527	2.67	3.45	29.2
528	2.41	2.99	24.1
532	2.06	2.32	12.6
537	3.32	4.04	21.7
545	2.17	2.66	22.6
550	1.93	2.54	31.6
558	5.32	6.22	16.9
3439	5.74	6.76	17.8
Average	3.21	3.90	21.4

The average increase observed in the urine collected from nine lambs was 21.4%. From these data it is apparent that a part of the nicotinic acid is excreted as conjugates. The increase in nicotinic acid values due to hydrolysis is, however, much less for sheep urine than that reported for human urine. Bandier ('39 b) found that alkaline hydrolysis of human urine gave values from 164 to 333% higher than unhydrolyzed urine. Swaminathan ('39) reported increases of from 45 to 106% after alkaline hydrolysis.

These investigators did not indicate whether or not their subjects were smokers. This may be a factor in accounting for difference in the relative amounts of free and conjugated forms of nicotinic acid excreted in man and sheep. Ackermann ('12)

found nicotinuric acid in the urine of a dog that had received nicotinic acid orally, while Harris and Raymond ('39) observed that a heavy smoker excreted approximately twice as much nicotinic acid per day as did non-smokers.

The relatively small increase in the nicotinic acid values after the hydrolysis of sheep urine affords no basis warranting the revision of the previous conclusions of the authors that nicotinic acid is synthesized in the alimentary tract or body of sheep. This conclusion was based on the fact that lambs restricted to a deficient diet continue to excrete in their urine essentially as much nicotinic acid as lambs on a standard well balanced growing diet. On the other hand dogs used to assay the deficient diet excreted in unhydrolyzed

TABLE 3

Recovery of nicotinic acid after administration of test dose

LAMB NUMBER	AMOUNT INGESTED	COLLECTION PERIOD	TOTAL EXCRETION	INITIAL EXCRETION	NET RECOVERY	PER CENT RECOVERED
	gm.	*hr.*	*mg.*	*mg.*	*mg.*	
523	8.20	73	7576	16.0	7560	92.2
532	6.20	36	3673	8.0	3665	59.1
537	10.45	50	4725	17.0	4708	45.1
550	8.38	50	3594	10.6	3583	42.7
558	9.94	50	7107	26.0	7081	71.2

samples of urine approximately fifteen times as much nicotinic acid daily on a stock diet as they did after being fed the experimental diet long enough to develop typical blacktongue symptoms.

Excretion of nicotinic acid following massive doses. The recovery of nicotinic acid in the urine after the ingestion of a single large dose was studied with five normal lambs that received a stock diet. The urine was collected for 3 consecutive days prior to the administration of the nicotinic acid. The nicotinic acid was given by mouth in gelatin capsules. The urine was collected for various periods after the ingestion of the test dose and the nicotinic acid determined on the hydrolyzed samples.

The data on the recovery of ingested nicotinic acid are summarized in table 3. The recovery of nicotinic acid in the urine

ranged from 42.7 to 92.2%. The recovery of nicotinic acid in the urine of sheep is considerably higher than has been reported for man when smaller amounts were ingested. Bandier ('39 b) fed 90 mg. and recovered 13.9% within 6 hours at which time the excretion had returned to normal. Swaminathan ('39) fed ten normal individuals each 100 mg. of nicotinic acid and recovered between 14.0 and 37.9% during the first 24 hours. Of 1 gm. of nicotinic acid administered subcutaneously to a rabbit Ritsert ('39) recovered from the urine 39.0% over a period of 120 hours. When the same amount was given orally 31.0% was recovered.

TABLE 4

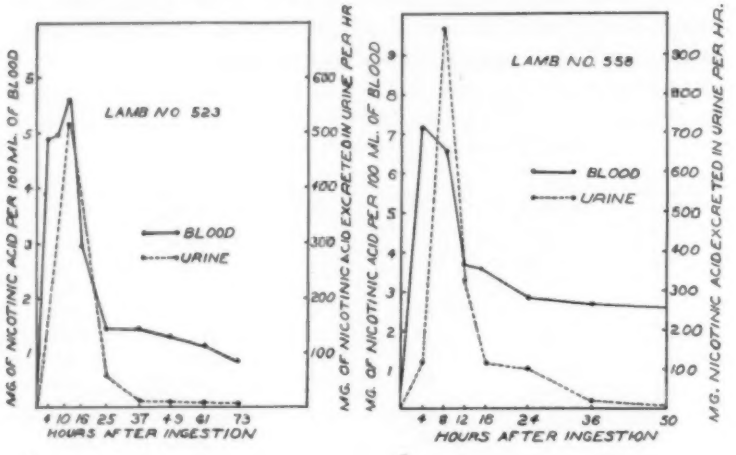
Rate of excretion of nicotinic acid after test dose

LAMB NUMBER	AMOUNT INGESTED	HOURS AFTER INGESTION	COLLECTION PERIOD	TOTAL EXCRETION DURING PERIOD	EXCRETION PER HOUR
	gm.		*hr.*	*mg.*	*mg.*
537	10.45	4	4	96	24.0
		8	4	1175	293.7
		12	4	887	221.7
		24	12	2137	178.1
		36	12	280	23.3
		50	14	150	10.7
550	8.38	8	8	1662	207.7
		16	8	977	122.1
		24	8	649	81.1
		36	12	166	13.8
		50	14	140	10.0

Rate of excretion. The urine of four of the lambs was collected at frequent intervals in order to obtain information on the rate of elimination of large doses of nicotinic acid. Originally it was planned to collect the urine at 4-hour intervals for the first 16 hours, followed by 8-, 12- and 14-hour periods. However, since micturition did not always occur during the 4-hour periods it was necessary to vary this plan. While micturition is more or less irregular especially when animals are confined to metabolism cages there is no reason to believe that renal secretion does not proceed at a relatively constant rate as under normal conditions. Thus in table 4 the rate

of excretion of nicotinic acid has been calculated on an hourly basis for the various collection periods. In order to conserve space the detailed data are shown in table 4 for only two of the lambs while the data for the other two are presented graphically in figures 1 and 2.

Of the total amount of nicotinic acid excreted during the 50 hours the urine was collected, lamb no. 550 excreted 46.2% during the first 8 hours. The comparable figures for animals



Figs. 1 and 2 Nicotinic acid content of the blood and urinary excretion of lamb nos. 523 and 558 following the ingestion of 8.20 and 9.94 gm. of nicotinic acid respectively.

558 and 537 are 60.8 and 26.9% respectively. The lower amount excreted by no. 537 is reflected in the higher concentration in the liver as will be brought out later. Lamb no. 523 did not micturate during the first 8 hours. The first collection of urine from this animal was made on the twelfth hour. During this period the excretion of nicotinic acid was 88.5% of the total for the first 49 hours. From this it appears that in sheep large doses of nicotinic acid are rapidly eliminated from the body by the kidney. This agrees with observations

of Ritsert ('39) who administered 1 gm. of nicotinic acid subcutaneously to a rabbit and during the first 24 hours recovered 85.1% of the total excreted over a 120-hour period. When the same amount was given by mouth 65.3% of the total excreted was recovered in the urine during the first 24 hours.

Free and conjugated nicotinic acid in urine following ingestion of massive doses. By determining the nicotinic acid values of urine on hydrolyzed and unhydrolyzed samples following the ingestion of a massive dose, information has been obtained on whether under such conditions nicotinic acid is excreted as the free acid or as conjugates. The effect of hydrolysis is shown in table 5. The total time during which

TABLE 5

Effect of alkaline hydrolysis on nicotinic acid values of urine after ingestion of massive dose

LAMB NUMBER	TOTAL NICOTINIC ACID EXCRETED		INCREASE FROM HYDROLYSIS
	Unhydrolyzed	Hydrolyzed	
	gm.	*gm.*	%
523	7.24	7.58	4.7
537	4.19	4.71	12.4
550	3.48	3.69	3.2
558	6.26	7.11	13.6

the urine was collected is the same as recorded for these animals in table 3. The hydrolyzed urine gave values of from 3.2 to 13.6% higher than the unhydrolyzed. Since nicotinamide gives only 20% of its maximum color if unhydrolyzed (Kodicek, '40) it appears that a part, though a relatively small percentage, of the ingested nicotinic acid was converted to some conjugated form in the body. A comparison of the data in tables 2 and 5 indicates that a much smaller percentage of a large dose of nicotinic acid is excreted as conjugates than occurs under normal conditions.

Relationship of blood and urinary nicotinic acid following massive dose. In connection with the study of the urinary excretion, blood was drawn at frequent intervals following

the test dose. The normal nicotinic acid level of the blood was determined while the animals were on the stock diet and prior to the administration of the test dose. The relationship of the blood nicotinic acid to the urinary excretion is shown graphically for two animals in figures 1 and 2. The rate of urinary excretion of nicotinic acid is calculated on an hourly basis for each collection period.

From the figures it is apparent that there is a close correlation between the urinary excretion and the concentration of nicotinic acid in the blood. The increases in the urinary levels are, of course, of a far greater magnitude than those in the blood. Similar relationships were observed with lambs nos. 537 and 550, but the detailed data have been omitted in order to conserve space. The increases in the nicotinic acid level of the blood above the normal level were 8.3, 3.9, 5.9 and 11-fold for lambs 523, 537, 550 and 558 respectively.

The rapid fall of the nicotinic acid level of the blood parallels closely the decline in the rate of urinary excretion. The blood values of lamb no. 558 did not return to normal until approximately 73 hours after the administration of the test dose. Fifty hours after the administration of the test dose to lamb no. 558 the nicotinic acid level of the blood was still 4.4 times the normal level. These observations are in agreement with the findings of Axelrod et al. ('40) that the coenzyme I content of the blood can be increased from 1.5 to 3-fold by feeding nicotinic acid.

Observations on the liver. Three lambs were killed by exsanguination 50 hours after ingestion of the test dose and the nicotinic acid of the liver determined by the method of Bandier ('39 a) with the modification that metol was replaced by aniline as in the blood and urine analysis. While the number of observations recorded in table 6 is too small to permit any conclusions they may serve to draw attention to points that might merit further study. During the 50-hour collection period lamb no. 550 excreted in the urine 42.7% of the ingested nicotinic acid. The liver contained 10.6 mg. of nicotinic acid per 100 gm. of fresh tissue. Histological examination showed

marked fatty degeneration of the liver which may account for its low nicotinic acid content. Since the livers of both of the other lambs appeared normal it is not believed that the fatty degeneration was caused by the massive dose of nicotinic acid. However, a final answer to this problem must await further studies. Forty-five per cent of the nicotinic acid ingested by lamb no. 537 was recovered in the urine, and the liver contained 44.0 mg. per 100 gm. of fresh tissue. The corresponding data for lamb no. 558 are 71.2% recovered in the urine and 23.4 mg. of nicotinic acid per 100 gm. of fresh liver. On the basis of the nicotinic acid content for normal sheep liver of 20.0 mg. per 100 gm. of fresh liver (Kodicek, '40) and values of the same order for ox and pork liver (Bandier, '39 a; Karrer and Keller, '39; Kringstad and Naess, '39) it appears that the ingestion of a single massive dose of nicotinic acid materially increases the nicotinic acid content of the liver. The extent to which the liver and other organs can be enriched in nicotinic acid by the ingestion of liberal amounts might merit investigation.

TABLE 6

Nicotinic acid content of livers

NUMBER	INGESTED	RECOVERED IN URINE	PER 100 GM. FRESH LIVER	TOTAL
	gm.	%	*mg.*	*mg.*
537	10.45	45.1	44.0	135.5
550	8.38	42.7	10.6	51.9
558	9.94	71.2	23.4	81.4

SUMMARY

There is no essential difference in the nicotinic acid level of the blood of lambs fed a diet low in nicotinic acid and those fed a stock diet. Lambs restricted to the deficient diet for several months continue to excrete in their urine approximately as much nicotinic acid as those on a stock diet.

Subjecting the urine of normal animals to alkaline hydrolysis increased the nicotinic acid values 21.5%. Since the in-

crease after the ingestion of a single large dose of nicotinic acid is much smaller, it appears that only a relatively small percentage is conjugated in the body under these conditions. Following the ingestion of a single large dose of nicotinic acid 42.7 to 92.2% was recovered in the urine.

Fifty hours after the ingestion of a large dose of nicotinic acid the amount in the liver was materially augmented. The extent of this increase appears to bear some relationship to the amount excreted.

Following the ingestion of a massive dose the nicotinic acid level of the blood increases severalfold. This rapid increment and the subsequent decline parallels closely the rate of urinary excretion.

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PANTOTHENIC ACID REQUIREMENT OF THE RAT

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THREE FIGURES

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It has long been recognized that rats maintained on synthetic diets supplemented with thiamin and riboflavin need, for normal growth and development, additional factors which are present in liver and yeast. Lepkovsky, Jukes and Krause ('36) separated from liver concentrates two fractions, one of which later proved to be vitamin B_6. The other fraction, factor II or filtrate factor, was found necessary for the growth of rats and was effective in curing the chick dermatitis, previously recognized as a vitamin deficiency by Elvehjem and Koehn ('35). Later, Jukes ('39 a, b) and Woolley, Waisman and Elvehjem ('39) reported that this chick antidermatitis factor was probably identical with pantothenic acid (Williams, '39). Progress in the preparation of pantothenic acid has made it possible to investigate its nutritional effect in rats.

Subbarow and Hitchings ('39) observed a marked growth response in rats, on a basal diet supplemented with the crystalline factors of the vitamin B complex, when fed a liver concentrate containing pantothenic acid. Oleson, Woolley and Elvehjem ('39) reported that the rat requires in addition to the available crystalline factors of the B complex, an alkali labile factor which is probably pantothenic acid. Recently György, Poling and Subbarow ('39) reported that a crude zinc salt of pantothenic acid was curative for skin lesions which developed on diets devoid of the filtrate factor.

In the Merck Research Laboratories, pantothenic acid was made from pure "natural" α-hydroxy-β, β-dimethyl-gamma-butyrolactone and β-alanine, and samples of it were made available by Doctor Stiller and Doctor Keresztesy for this study. The potency of the preparation was determined by its growth stimulating effect on Lactobacillus casei (Snell, Strong and Peterson, '37) in direct comparison with a pantothenic acid standard furnished by Dr. R. J. Williams. Pantothenic acid made by such a procedure is free from any other nutritional factors which might be present as impurities in natural pantothenic acid concentrates.

The animals used in these experiments were maintained on a vitamin B complex free diet consisting of dextrose, 68%; casein, 18%; hydrogenated cottonseed oil,[1] 8%; salt mixture, 4%; cod liver oil, 2% and supplemented with 40 μg. each of thiamin, riboflavin and vitamin B_6 and 5 mg. of choline chloride per rat per day. In part of the experiments nicotinic acid amide was also added in daily doses of 0.5 mg.

Twenty-one-day-old rats placed on this regimen grew at a rate of approximately 1.5 gm. per day throughout the first 3 weeks, at which time the weight became stationary at 50 to 65 gm. If no pantothenic acid was administered, the survival time on this diet was from 25 to 60 days. Starting at the second week, the rats developed a rough, thin fur, excessive nasal secretion, rusty spots on the fur, sores around the mouth and blood-caked whiskers. Change in the appearance of the fur was invariably the first symptom. The long hairs of the cover coat became loose and were readily shed. The fur lost its lustre and took on the appearance of that of a very young animal. Thinning of the soft short hair occurred to such an extent that blowing at the fur readily exposed the bare, rough and scaly skin. This process started usually at the groin and on the head just back of the ears, but soon became generalized over the body. Some animals approached complete baldness. Excessive nasal secretion occurred in the

[1] Crisco.

majority of the animals and was occasionally found to contain blood. Sores around the mouth frequently developed. The rats showed rusty spots on the fur especially on the head and on the back. During the third week the whiskers showed some bloody discoloration which later developed into blood-tinged cakes on the whiskers. It cannot be stated definitely whether the blood on the whiskers was caused by bleeding into the hair follicles or by the animals rubbing it onto the whiskers from sores around the mouth. The phenomenon of blood-caked whiskers occurred in the majority of the animals, sometimes to such an extent that the rat assumed the bearded appearance of a walrus. Deficiency symptoms on the eyelids were observed in only 10 to 20% of the animals. They began with an exudation on the lids which kept the eye stuck shut. Later these animals showed denudation around the eye, a halo resembling spectacles. In addition to the findings noted above, a large percentage of the rats showed, on autopsy, numerous petechiae under the skin and a hemorrhagic condition of the adrenals and intestines. Histopathological studies are in progress and will be reported later.

Curative effect following single doses

Single doses of 100, 200, 300, 600 and 840 μg. respectively of pantothenic acid were administered orally to groups of six to ten rats which had been on the deficient diet for 30 to 40 days and exhibited the above described symptoms. Daily records of weight and symptoms were kept in all experiments.

A single dose of 100 μg. was without noticeable effect. The response to single doses of 200, 300 and 600 μg. was irregular. About half of the animals on each of these dose levels gained 1 to 2 gm. per day over a period of 3 to 4 days; the other half failed to show any significant weight gain and died probably because single doses within this range were not sufficient to influence a well advanced deficiency. However, with a single dose of 840 μg. only three out of ten rats died and the remaining animals showed a uniform growth response of about 3 gm.

per day for 4 to 5 days. Growth then slowed down and the weight curve leveled off. A second dose of 840 μg., administered 6 days after the first, was followed by an almost identical response. Again the weight became stationary after 6 days. A third dose of 840 μg. administered on the fifteenth day produced a repetition of the effect of each of the previous doses (fig. 1).

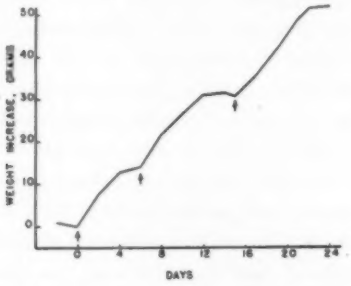


Fig. 1 Effect of single doses of pantothenic acid on the growth of rats depleted of pantothenic acid on a vitamin B complex free diet supplemented with 40 μg. of each, thiamin, riboflavin, and vitamin B_6, 0.5 mg. of nicotinic acid, and 5 mg. of choline chloride per rat per day. At each arrow, 840 μg. of pantothenic acid were given orally.

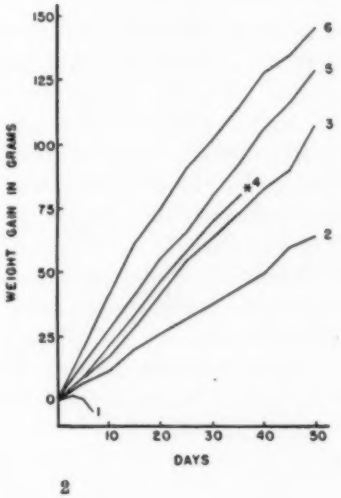
Curative effect following daily feeding

To rats which had reached a stationary weight after a depletion period from 19 to 34 days, daily doses of 12, 25, 50, 100 and 150 μg. of pantothenic acid respectively were administered orally. Groups of ten rats were used for each dose level. Another group received a daily supplement of 0.25 cc. of an aqueous extract of liver [2] containing 150 μg. of pantothenic acid as determined by bacterial assay. This supplement presumably supplies other as yet unidentified factors of the B complex.

In contrast to the administration of single doses, the response to daily feeding was much more uniform (fig. 2). At

[2] Kindly prepared and assayed for its pantothenic acid content by Dr. J. C. Keresztesy.

the 12 μg. level the average weight of the group declined below the starting level and all animals died within 16 days (curve 1). At the 25 μg. level 50% of the animals died within 2 weeks; the remaining rats, however, gained continuously at a rate of 1.25 gm. per day over the 50-day period of the test (curve 2). Of the animals receiving 50, 100 and 150 μg. respectively of pantothenic acid or its equivalent in the form of liver concentrate, 70% survived in each group. One hundred and fifty



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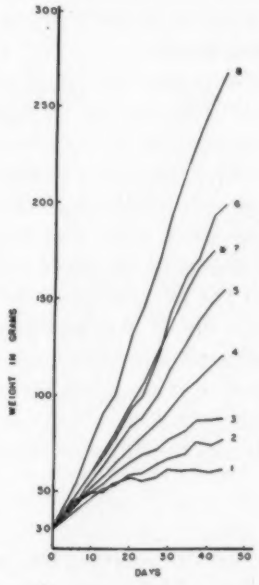

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Fig. 2 Growth response of pantothenic acid deficient rats to daily feeding of graded doses of pantothenic acid. (1) 12 μg.; (2) 25 μg.; (3) 50 μg.; (4) 100 μg.; (5) 150 μg.; (6) 0.25 cc. of a liver concentrate, containing 150 μg. of pantothenic acid. * Animals killed for autopsy.

Fig. 3 Effect of daily feeding of pantothenic acid on the growth of rats maintained on a vitamin B complex free diet supplemented with 40 μg. of each, thiamin, riboflavin, and vitamin B_6, 0.5 mg. of nicotinic acid, and 5 mg. of choline chloride. (1) No pantothenic acid; (2) 5 μg.; (3) 10 μg.; (4) 20 μg.; (5) 40 μg.; (6) 80 μg.; (7) 150 μg.; (8) 10% liver added to the diet containing 150 μg. of pantothenic acid per gram. * Animals killed for autopsy.

micrograms daily produced an average weight gain of 2.5 gm. per day (curve 5). This response was definitely greater than that following daily administration of 50 or 100 μg. (curves 3 and 4). Feeding the liver preparation containing 150 μg. of pantothenic acid per dose caused an average weight gain of 3 gm. per day (curve 6), which exceeds the response to 150 μg. of pantothenic acid. While we agree with Woolley ('40) that the administration of liver produces a greater weight gain than that of pantothenic acid alone, our findings with this latter substance are contrary to his, inasmuch as growth is continuous.

Whereas the effect of single doses was of too short duration to influence the deficiency symptoms significantly, definite changes were observed in the animals receiving pantothenic acid daily over a period of 50 days. At the end of the test only one out of seven animals receiving 150 μg. daily still exhibited excessive nasal secretion and some blood on the whiskers. The fur of all animals in this group was less coarse and rough than at the beginning of the test, but still was lacking the lustre of the fur of rats on complete diets. About the same regression of symptoms was found in the group receiving the liver preparation containing 150 μg. of pantothenic acid. In the groups receiving 25 and 50 μg. daily, no definite improvement in external appearance could be observed during the 50-day period.

Prophylactic test

In this experiment a total of ninety 3-week-old male rats, divided into nine groups of ten each, were placed on the same dietary regimen as is described on page 566. They were further supplemented as follows: One group receiving no pantothenic acid served as a negative control. A second group receiving whole dried beef liver [3] mixed with the diet to an extent of 10% served as a full diet control. The pantothenic acid content of the liver, as determined by the bacterial growth

[3] Kindly prepared and assayed for its pantothenic acid content by Dr. J. C. Keresztesy.

method, was sufficient to supply 150 μg. per 10 gm. of diet. To the remaining groups various doses of pantothenic acid were administered daily orally. One of the groups on 150 μg. daily did not receive nicotinic acid amide. The average weight responses to the daily feeding of graded doses of pantothenic acid are recorded in figure 3. The animals receiving no pantothenic acid reached stationary weight after 30 days on the test diet (curve 1). The group receiving 5 μg. daily likewise ceased to gain about the thirty-seventh day but attained a slightly higher weight than the negative control group (curve 2). Ten micrograms daily were still insufficient to produce continuous growth, the weight becoming almost stationary at 90 gm. (curve 3). Higher doses produced continuous growth in the rats throughout the test period as evidenced by curves 4, 5 and 6 in figure 3. No significant difference was found between the growth responses to 80 and 150 μg. of pantothenic acid daily. The average weight gain per rat per day obtained with these doses was 4 gm. On the other hand, the rats receiving dried liver gained weight at an average of 5.3 gm. per day. The group receiving 150 μg. of pantothenic acid but no nicotinic acid amide grew at a somewhat slower rate than the corresponding group with nicotinic acid amide.

The manifestations noted in the animals receiving no pantothenic acid have already been described. Daily doses of 5 or 10 μg. of pantothenic acid failed to produce any improvement in appearance as compared with the negative control group. Twenty micrograms daily, while sufficient to prevent death and to allow continuous growth throughout the test period, likewise failed to influence appreciably the deficiency symptoms. The animals receiving 40 μg. daily developed a much better coat, definitely less thin and coarse than that of the animals on the lower dose levels of pantothenic acid, but still lacking the smoothness and the lustre of normal fur. In this group no blood-caked whiskers were observed, and only a few animals showed rusty spots on the fur. On the forty-third day of the test, these appearances seemed to be in regression. On a daily dose of 80 μg. of pantothenic acid the animals did not

exhibit any external symptoms except for a short period between the third and fifth weeks, when the coat was somewhat rough and not in as good condition as that of the animals receiving 150 μg. of pantothenic acid or the liver supplement. This manifestation, however, was transient; after the thirty-fifth day the fur of all animals receiving 80 μg. matched that of the animals receiving liver. With the exception of their respective weights, no significant difference could be found at

TABLE 1

Effect of pantothenic acid on the incidence of deficiency symptoms observed in rats maintained on a vitamin B complex free diet supplemented with 40 μg. of each, thiamin, riboflavin, and B_6, 0.5 mg. of nicotinic acid, and 5 mg. choline chloride per rat per day

DAILY DOSE OF P.A. IN MICRO-GRAMS	PER CENT SURVIVAL AFTER 6 WEEKS	STATIONARY WEIGHT REACHED ON	COARSE THIN, FUR	BLOOD-CAKED WHISKERS	RUSTY SPOTS	PER CENT OF SPECTACLED EYES	PER CENT OF ADRENAL HEMORRHAGES
0	40	30th day	++	++	++	20	40
5	40	37th day	++	++	++	0	20
10	90	50th day	++	++	+	10	10
20	100		++	++	++	0	0
40	100		+	0	+	0	0
80	100		(+)	0	0	0	0
150	100		0	0	0	0	0
150 in liver	100		0	0	0	0	0

the end of the test period in the appearance of the rats receiving 80 or 150 μg. of pantothenic acid and those on the liver supplement. In table 1 an attempt has been made to summarize the incidence of the various symptoms in the different groups.

DISCUSSION

Effect on growth

Since the substance used in our experiments was pantothenic acid obtained by condensation of the pure "natural" lactone and beta-alanine, it seemed advisable to study the effect of each of the constituents of the molecule. Beta-alanine alone administered to groups of ten deficient rats in single doses of 1.8 and 9.3 mg. respectively was without any significant effect,

confirming the findings of El-Sadr et al. ('39), Oleson, Woolley and Elvehjem ('39), and György, Poling and Subbarow ('39). Daily feeding of 0.5 mg. of either beta-alanine, the lactone or both together over a period of 40 days to groups of ten young rats on the basal diet did not increase the growth rate over that of the negative control group. Thus, it is evident that the rat cannot utilize the components of pantothenic acid singly or when mixed together, but needs the intact pantothenic acid molecule. Hence, the effects obtained in our experiments must be attributed to the administration of pantothenic acid per se.

Young rats maintained on the basal diet supplemented with the synthetic crystalline factors, attained a growth rate of 4 gm. per day when fed with 80 μg. of pantothenic acid per day. Larger doses did not increase this figure. However, the addition of 10% of dried liver to the diet (equivalent to feeding 150 μg. of pantothenic acid per 10 gm. of food) produced a growth rate in excess of that found with pantothenic acid alone. These findings demonstrate the important role of pantothenic acid as a growth stimulating substance and at the same time, offer further evidence for the presence of additional growth stimulating factors in liver.

Effects on external symptoms

Of the deficiency symptoms manifested on our diet, almost every one has been previously observed either singly or in combination in animals on diets devoid of the "filtrate factor." It should be kept in mind that the diets used by different investigators varied greatly in the percentage and character of their constituents and supplements, a fact which might exert considerable influence on the time, appearance, incidence and degree of severity of the lesions. Hence, comparisons between our findings and those of other investigators can only be tentative.

The most consistent symptom observed in our experiments was the change in the condition of the fur. György and Eckhardt ('39) record, in some of their deficient animals, an

alopecia extending over the neck and back, but give greater emphasis to the scaliness and inflammation of the skin. Lepkovsky, Jukes and Krause ('36) mention dermatitis; and Chick, Macrae and Worden ('40) emphasize the characteristic change in the fur of animals deficient in "filtrate factor."

Excessive nasal secretion occasionally containing blood was observed in the majority of our animals. "Inflammed nose" was recorded by Lepkovsky, Jukes and Krause ('36) in rats lacking the filtrate factor, and recently Daft and Sebrell ('39) found nose bleed a predominant sign in rats on a B complex diet but supplemented with the crystalline factors.

No mention of blood-caked whiskers, which we observed in the majority of our animals, could be found in the recent literature. Whether this symptom and the rusty spots or "browning" (Lunde and Kringstad, '39) of the hair is caused by the animals rubbing blood from the nose or from sores around the mouth onto the fur and the whiskers is not as yet established.

In agreement with the statement by György and Eckhardt ('39) "spectacled" eyes were found in only a small percentage of our animals. The description given by Lepkovsky, Jukes and Krause ('36) and Lunde and Kringstad ('39) holds good for the signs as observed on our diet, except that no erosions (Oleson, Bird, Elvehjem and Hart, '38; Oleson, Elvehjem and Hart, '39) were found around the eye.

Autopsies on our deficient rats confirmed the findings of Daft and Sebrell ('39) and Nelson ('39) on adrenal cortical hemorrhages.

At the present time it cannot be decided definitely whether all or some of the symptoms are specifically due to lack of pantothenic acid in the diet. Although the administration of pantothenic acid produces an improvement of the external symptoms, chiefly noticeable in the condition of the fur, the response was rather delayed and high doses over a prolonged period of time had to be administered to demonstrate a curative effect. This contrasts sharply with the dramatic improvement of acrodynia lesions following the administration of vitamin B_6.

On the other hand, a close relationship between pantothenic acid and the deficiency syndrome becomes obvious from the results of the prophylactic test, summarized in table 1. None of the manifestations occurred on daily administration of 150 μg. of pantothenic acid, a dose above the requirement for maximal growth. With 80 μg. daily only fleeting signs of fur impairment were observed. Particularly, no adrenal hemorrhages were found with doses which assured continuous growth.

ACKNOWLEDGMENT

The valuable technical assistance of Miss Grace Richards and Miss Evelyn Ruddy is gratefully acknowledged.

SUMMARY[4]

The effect of pantothenic acid prepared from pure "natural" α-hydroxy-β, β-dimethyl-gamma-butyrolactone and β-alanine has been studied on rats maintained on a synthetic diet supplemented with the synthetic crystalline factors of the B complex.

1. Rats maintained on a diet devoid of pantothenic acid cease to grow after 3 to 4 weeks and develop a deficiency syndrome characterized by scant, coarse fur showing rusty spots, inflammation of the nose, and blood-caked whiskers. On autopsy some of the rats show hemorrhages under the skin and into the adrenal cortex.

2. A single dose of 800 μg. or daily feeding of 50 μg. of pantothenic acid produces a marked weight response in deficient rats, but the deficiency symptoms yield only after prolonged administration.

3. In prophylactic tests the maintenance dose for optimal growth is approximately 80 μg. of pantothenic acid per rat per day.

4. Liver concentrates of known pantothenic acid content are superior to equal doses of pantothenic acid alone in their growth-promoting effect.

[4] While this study was in progress, synthetic pantothenic acid (Williams and Major, '40) became available. Using calcium pantothenate we have duplicated all of our findings on the curative effects reported above.

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THE EFFECT OF VITAMINS A AND C ON EXPERIMENTAL HYPERTHYROIDISM [1]

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TWO FIGURES

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Since 1932, numerous reports have appeared in the literature pertaining to the antagonism demonstrated by vitamin A or its precursor, carotene, to thyrotoxicosis (Murlin, '38). During thyrotoxicosis, von Euler and Klussmann ('32) found vitamin A stores of the liver to be depleted, and that carotene would counteract the body weight loss associated with this condition. According to Eufinger and Gottlieb ('35) vitamin A will depress the acceleration of metamorphosis of tadpoles induced by thyroxin. Rappai and Rosenfeld ('35) demonstrated that the hypermetabolism produced by thyroxin injections can be reduced to some extent by vitamin A therapy, but the increased metabolism induced by dinitrophenol, according to the experiments of Logaras and Drummond ('38), will not be affected. Abnormalities of carbohydrate metabolism associated with hyperthyroidism have been reported by David ('38) and by Egelin ('39) to be reduced to a minimum by vitamin A. From the work of Fasold and Heidemann ('33) it seems that the thyroid is necessary in some way for the conversion of carotene to vitamin A. This was evident after they noted that thyroidectomy changed the composition of

[1] This investigation was supported by a grant of the National Research Council's Committee on Endocrinology.

[2] The data in this paper were taken from a thesis presented in partial fulfillment of the requirements for the doctorate of philosophy, University of Rochester, June, 1940.

goat milk from its usually high vitamin A content to one high in carotene. Wohl and Feldman ('39) bear out this relationship in their findings of pathological dark adaptation amongst their cases of hypothyroidism. With increased metabolism, Sure and Buchanan ('37) have shown that there is an increased requirement of vitamin A.

Independently, Demole and Ippen ('35) and Oehme ('36) reported ascorbic acid capable of reducing the hypermetabolism of thyrotoxicosis. Creatinuria of a hyper-thyroid animal or human can be suppressed by ascorbic acid therapy, as shown by Fischer and Oehme ('37) and by v. Plehwe ('38). The experiments of Marine, Baumann and Rosen ('34) as well as those by Schulze and Linnemann ('38) point to the idea that vitamin C is able to inhibit the action of thyrotropic hormone on the thyroid gland. The blood picture presented by the scorbutic guinea pig resembles that of an hyperthyroid animal (Aszódi, '37).

PROCEDURE

Rats used in this investigation were of the Wistar strain. Only one major group of animals of similar age and sex was utilized for the study of the efficacy of ascorbic acid in counteracting the hypermetabolism of hyperthyroidism. Two major groups of rats were used in the vitamin A studies. Each group was subdivided into: (1) control rats which were on the stock diet of commercial dog food,[3] (2) rats receiving only vitamin A or ascorbic acid in addition to the stock diet, (3) animals receiving thyroxin injections, and finally (4) rats receiving vitamin A or ascorbic acid in addition to the thyroxin injections.

Crystalline thyroxin [4] which was dissolved in N/100 NaOH, was injected subcutaneously in the amount of 0.2 mg. per 100 gm. of body weight daily over a period of 6 successive days. The animals were weighed daily and the dose of thyroxin was adjusted accordingly.

[3] Purina dog chow.
[4] Squibb.

The vitamin A concentrate was given orally in the amount of 2000 U.S.P. units/day over a period of 8 days. The animals receiving this concentrate along with the thyroxin injections (6 days), received their vitamin A supplement 2 days previous to the initiation of the thyroxin administration. This permitted the animal to build up its vitamin A stores. Ascorbic acid was injected subcutaneously in the amount of 20 mg. per 100 gm. of body weight per day for 6 successive days. In experiments employing both vitamin C and thyroxin the two were administered over the same length of period.

On the sixth and last day of injection, the basal metabolism was determined on the rats in the post-absorptive condition. The apparatus used for such determinations employed the Haldane principle, moisture, carbon dioxide production and oxygen consumption being determined gravimetrically. This particular form of apparatus was constructed similarly to the one employed by Forbes, Kriss and Miller ('34) and was the same apparatus used by Black ('39). All measurements were made with the temperature of the respiration chamber between 28°C. and 29°C. The length of the experimental period was 6 hours. Basal heat production values are expressed as Calories per square meter of body surface per hour, according to the formula of Diack ('30).

On the seventh day the animal still in the post-absorptive state was sacrificed by a blow on the head, and the liver, kidneys and thyroid removed as quickly as possible. The tissues were sliced with a straight sectioning razor. The respiration of these tissues was determined by means of the Warburg constant volume manometers utilizing the Marsh ('37) type of respiration flask. This type of flask permitted the determination of Q_{O_2}, Q_{CO_2} and consequently the respiratory quotient in the same flask, although appropriate controls were necessary. Both kidney cortex and liver tissue slices were run in triplicate, but only single determinations of thyroid respiration (Q_{O_2}) were made, since the thyroids of rats are quite small. The tissue medium used was a Ringer-

TABLE 1

Basal metabolism data of vitamin studies in experimental thyrotoxicosis

	SEX	AGE	NO. IN GROUP	CAL./HR.	PER CENT CHANGE	CAL./KG./HR.	PER CENT CHANGE	CAL./M²/HR.	PER CENT CHANGE	R.Q.
		(*months*)								
Controls	F	6	6	0.894	....	5.009	....	36.9	....	0.73
Controls receiving vitamin C	F	6	4	0.852	—5	4.889	—4	35.9	—3	0.74
Thyroxin treated	F	6	5	1.350	+51	8.173	+63	58.7	+59	0.73
Thyroxin treated + vitamin C	F	6	5	1.180	+32	6.949	+39	49.8	+35	0.73
Controls	M	4	6	1.156	....	4.698	....	38.5	....	0.73
Controls receiving vitamin A	M	4	6	1.156	0	4.710	0	38.6	0	0.73
Thyroxin treated	M	4	6	1.933	+67	9.920	+111	75.2	+95	0.73
Thyroxin treated + vitamin A	M	4	5	1.943	+68	7.789	+66	64.1	+66	0.73
Controls	F	18	6	1.001	....	4.453	....	35.4	....	0.73
Controls receiving vitamin A	F	18	5	1.041	+4	4.893	+9	38.5	+9	0.73
Thyroxin treated	F	18	6	1.652	+65	7.777	+75	60.4	+71	0.73
Thyroxin treated + vitamin A	F	18	6	1.528	+53	7.045	+58	55.5	+57	0.74

TABLE 2

Tissue respiration data of vitamin studies in experimental thyrotoxicosis

	SEX	AGE	NO. IN GROUP	LIVER					KIDNEY					THYROID	
				Q_{O_2}	Per cent change	Q_{CO_2}	Per cent change	R.Q.	Q_{O_2}	Per cent change	Q_{CO_2}	Per cent change	R.Q.	Q_{O_2}	Per cent change
		(months)													
Controls	F	6	6	7.05	....	3.65	....	0.52	17.3	....	14.5	....	0.84	4.14	....
Controls + vitamin C	F	6	4	8.11	+15	3.91	+7	0.48	19.4	+12	16.1	+11	0.83	5.13	+24
Thyroxin treated	F	6	6	8.46	+20	4.12	+13	0.49	19.0	+10	15.8	+9	0.83	2.60	—37
Thyroxin treated + vitamin C	F	6	5	8.72	+24	4.06	+11	0.46	20.1	+16	17.0	+17	0.84	2.70	—35
Controls	M	4	6	7.07	....	3.94	....	0.56	17.2	....	13.9	....	0.81	5.35	....
Controls + vitamin A	M	4	6	7.52	+6	3.79	—4	0.50	18.3	+6	15.2	+9	0.83	4.83	—10
Thyroxin treated	M	4	6	8.69	+23	4.38	+11	0.51	19.1	+17	15.4	+11	0.81	3.33	—38
Thyroxin treated + vitamin A	M	4	5	9.62	+36	4.63	+18	0.49	20.5	+19	16.8	+21	0.82	2.75	—49
Controls	F	18	6	7.01	....	3.35	....	0.47	17.0	....	13.7	....	0.81	3.74	....
Controls + vitamin A	F	18	5	7.63	+9	3.75	+12	0.49	17.0	0	13.2	—3	0.78	3.37	—10
Thyroxin treated	F	18	6	9.26	+32	4.49	+34	0.48	20.6	+21	16.8	+23	0.82	2.63	—30
Thyroxin treated + vitamin A	F	18	6	9.38	+34	4.46	+32	0.47	19.9	+17	15.7	+15	0.79	2.75	—26

phosphate-glucose solution, the gas space in the respiration flask being filled with air (Belasco, '40).

RESULTS

Summaries of the basal metabolism and tissue respiration data are shown in tables 1 and 2 and in figures 1 and 2. Vitamin C injections alone did not alter the basal heat production of the 6-month-old females. When ascorbic acid was

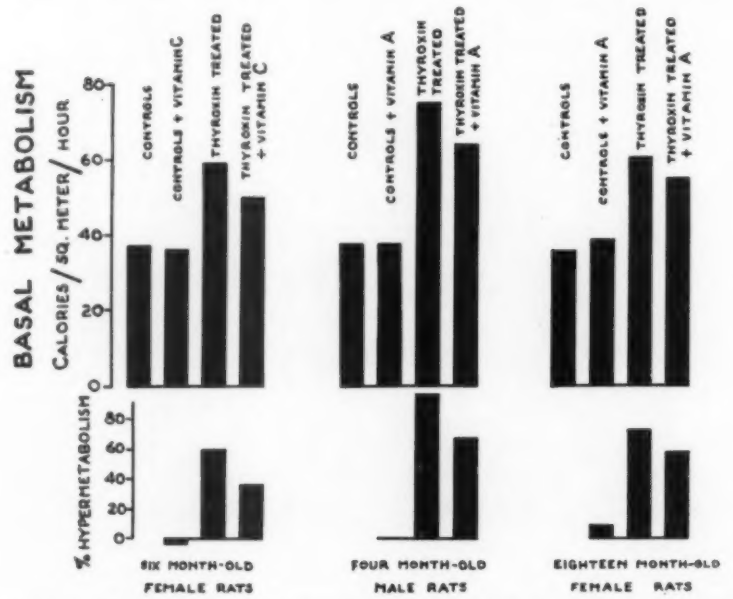

Fig. 1 The effects on the energy metabolism of rats of vitamins A and C administered with and without thyroxin. Note the effect of the vitamins in decreasing the hypermetabolism due to thyroxin.

given in conjunction with thyroxin injections, the resulting hypermetabolism was much less than the increased metabolism due to thyroxin alone. Thyroxin, by itself, produced an increase in heat production of 59%; this hypermetabolism was reduced to only 35% when ascorbic acid was given simultaneously with thyroxin.

From the point of view of the tissue metabolism data, this antagonism was not evident. Ascorbic acid administration alone caused increased liver, kidney and thyroid respiration. When thyroxin was injected simultaneously with vitamin C, the metabolism of liver and kidney tissue was even greater than would have resulted, if thyroxin alone were injected. Vitamin C in no way alleviated the depressed thyroid respiration that was produced by thyroxin treatment.

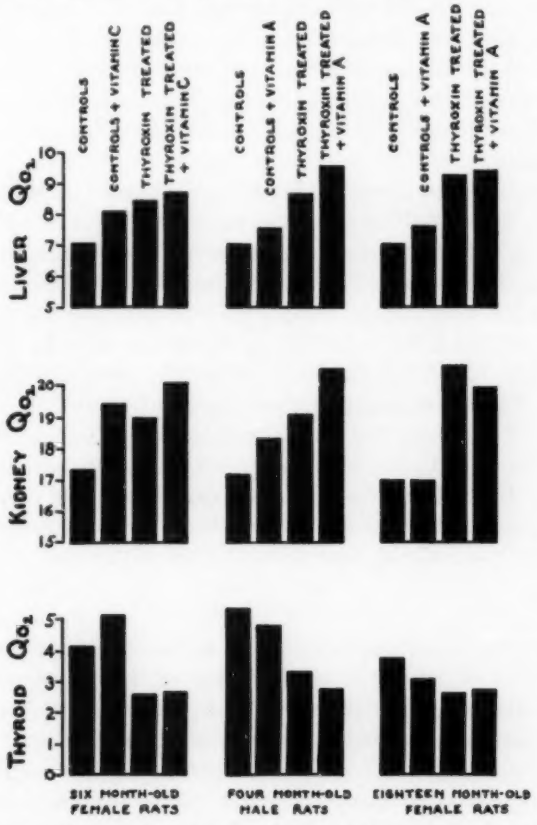

Fig. 2 The effect on the respiration of liver, kidney and thyroid of vitamins A and C administered with and without thyroxin.

Feeding the vitamin A concentrate alone did not alter the basal metabolism significantly. The 4-month-old males receiving this vitamin concentrate had exactly the same basal heat production as their controls. On the other hand, the metabolism of 18-month-old females seemed to be increased to the extent of 9% by vitamin A ingestion. The variation cannot be considered as significant at present because of the small number of animals used. The hypermetabolism of the 4-month-old males receiving thyroxin was 95% above normal basal metabolism. Treatment of thyrotoxicosis with vitamin A resulted in an increased metabolism of only 66%. The hypermetabolism of the thyroxin-treated 18-month-old female rats was reduced from +71 to +57 by the feeding of vitamin A concentrate.

The response of liver, kidney and thyroid respiration to vitamin A resembled the tissue respiration of thyroxin-treated animals, but to a lesser degree; that is to say, vitamin A stimulated hepatic and renal tissues to higher metabolic levels in the 4-month-old males, while it depressed the metabolism of thyroid tissue. In the aged females the vitamin stimulated only the liver tissue. Comparing the metabolism of tissues of rats receiving thyroxin and of those receiving the vitamin A concentrate along with thyroxin, no antagonism was evident at all. In fact, the liver metabolism was increased and the thyroid respiration decreased still further in the 4-month-old males. In the 18-month-old females this additive effect was very slight, and present only with liver.

DISCUSSION

There was a definite antagonism between both vitamins and thyroxin as evidenced by the lowering of the hypermetabolism of animals receiving either of the vitamins in conjunction with thyroxin. The tissue respiration data give no indication of this antagonism with either vitamin. Both vitamin A and ascorbic acid increased liver and kidney tissue respiration in the young rats. The point of difference between the two vitamins, in this regard, is that vitamin A depressed thyroid

oxygen uptake to the extent of 10%, while vitamin C increased the metabolism of the same gland 24%. These vitamins given in conjunction with thyroxin either did not affect the oxygen consumption of liver and kidney tissue derived from the thyrotoxic animal, or they increased the respiration of these tissues still further. Although the vitamin A concentrate alone reduced the thyroid oxygen consumption of thyroid tissue, it produced an enlargement of the same gland. This probably signifies that vitamin A in some way affects the pituitary or the thyroid gland itself, to cause a cessation of colloid excretion, thus producing a storage of the colloid in the follicles of the gland.

The effect of vitamin C on tissue metabolism in producing increased oxygen consumption is odd in the light of the finding of Stotz et al. ('37) that the liver respiration of scorbutic animals is higher than that of normals. Thus it seems that an excess or a deficiency of ascorbic acid will produce similar results. However, it is plausible to suppose that conditions leading up to increased respiration are not the same in both instances. In the scorbutic animal there is a deficiency of a hydrogen acceptor. In an attempt of the body to maintain its normal level of metabolism, the pathways of oxidation are re-routed, the change leading to less efficient metabolism with consequent increased oxygen consumption. From the other side, it is possible that a relative increase in a dehydrogenase, like ascorbic acid, might tend to bring about an increased oxygen consumption of normal tissues. Cohen and Gerard ('37) are of the opinion that the increased brain tissue metabolism caused by hyperthyroidism is associated with a relative increase of dehydrogenase concentration in the cells as compared to the oxidase system.

In relation to the stimulating effect of vitamin A on liver respiration, Ruddy ('39) recently observed that the liver tissue of vitamin A-deficient rats has an abnormally low oxygen consumption; but upon giving the animal larger amounts of vitamin, liver metabolism rose back to normal and even to a slightly higher level. No comment was made as to why vitamin

A would tend to increase tissue respiration. Heart tissue of a vitamin A deficient rat is known to have a low oxygen consumption. The increased tissue respiration of liver at least, and of kidney in the young male, produced by vitamin A ingestion is large enough to be reckoned with, for all results were obtained in triplicate. It appears that liver and kidney tissue in an effort to catabolize the excess amounts of vitamin A must necessarily have a higher metabolism.

No doubt there is an increased requirement of vitamin A and vitamin C in hyperthyroidism. If these increased requirements are not supplied, thyrotoxicosis becomes the more evident, thus presenting a picture of a more severe degree of hyperthyroidism than is produced by the thyroxin per se. Increasing the vitamin intake under such conditions therefore gives one the idea of a reduction in the degree of hyperthyroidism, while in actuality it may be only the removal of the deleterious effects of vitamin deficiency in this state of toxicity. However, vitamin A in some way antagonizes the active thyroid principle, possibly inhibiting thyroid function, as is evidenced by its ability to depress thyroid tissue respiration, but not that of liver or kidney. Possibly vitamin A, by virtue of its double bonds, is able to take up the available iodine in body tissues, and as an organic iodine compound to antagonize hyperthyroidism as do inorganic or organic iodine compounds, exclusive of thyroxin or thyroglobulin. Either by acting on the anterior pituitary gland or on the thyroid directly, this iodinated vitamin A may affect the storage of colloid and consequently cause lowered oxygen uptake of the thyroid. This antagonism will have to be studied more closely in the future to uncover the true mechanism.

SUMMARY

The basal metabolism and liver, kidney and thyroid tissue respirations of rats were studied to determine whether vitamin A and ascorbic acid could be shown to affect thyrotoxic tissue respiration as they do thyrotoxic basal metabolism. The effect of the vitamins alone was also studied.

Vitamin A and ascorbic acid did reduce the hypermetabolism associated with hyperthyroidism to some extent. Only in one group of rats, the 18-month-old females, did vitamin A raise the metabolism slightly. In the other groups neither vitamin produced any alteration of normal basal heat production.

Both ascorbic acid and vitamin A increased the respiratory rates of liver and of kidney cortical tissue. Ascorbic acid increased thyroid tissue respiration, while vitamin A depressed it. Increased liver and kidney tissue metabolism associated with hyperthyroidism was not altered by vitamins A or C. The depressed thyroid respiration, as a result of thyroxin administration, was not alleviated either by vitamin A or ascorbic acid. In fact, there were instances where the effect was additive, i.e., of the hormone plus the vitamin.

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THE INFLUENCE OF EXERCISE ON THE GROWING RAT IN THE PRESENCE AND ABSENCE OF VITAMIN B_1[1]

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FIVE FIGURES

(Received for publication August 12, 1940)

In a former communication from this laboratory (Guerrant, Dutcher and Chornock, '39) it was shown that when carefully selected groups of young rats were used as experimental subjects under comparable conditions of experimentation, less food was consumed, smaller increases in body weight were made and less severe symptoms of vitamin A deficiency developed when the animals were forced to exercise than when permitted to exercise voluntarily or when confined in the usual type of cage. It was shown that, although animals which were forced to exercise exhibited the greatest efficiency of food utilization, they voided the greatest number of fecal pellets, indicating a beneficial effect of exercise on intestinal motility. Furthermore, it was shown that animals, which were exercised voluntarily, and which received daily allotments of vitamin A, were more active physically than were the litter mates which did not receive the vitamin supplement.

The investigation reported at this time concerns itself with similar studies regarding the effect of exercise on vitamin B_1 requirement.

[1] Authorized for publication on July 31, 1940, as paper no. 982 in the Journal Series of the Pennsylvania Agricultural Experiment Station. Presented before the division of biological chemistry at the Cincinnati meeting of the American Chemical Society, April 10, 1940.

EXPERIMENTAL

The present investigation consisted essentially of a comparison of the physiological activity and the growth responses of comparable groups of young rats while being maintained under identical conditions, with the exception of the amount of exercise permitted by the type of caging employed and of the vitamin B_1 intake. The experimental equipment was identical with that used in the earlier experiments. With the exception of the following details, the experimental procedure was also similar to that used in the previous investigation.

Healthy rats, 23 to 26 days of age and weighing approximately 52 gm., were used as the experimental subjects. All animals were fed, ad libitum, a vitamin B_1 deficient basal diet which had the following percentage composition: purified casein 18, salt mixture 4, cellu flour 2, sucrose 71, filtered butterfat 3 and cod liver oil 2. Each animal received, daily, 10 μg. of riboflavin and the equivalent of 100 mg. of rice polish concentrate [2] as supplements to the basal diet. Each set of animals was divided into two groups. The animals of one group received the unsupplemented vitamin B_1 deficient diet until they were depleted of their body reserves of vitamin B_1. With the development of the paralytic symptoms characteristic of vitamin B_1 deficiency, each animal of this group was fed 6 μg. of vitamin B_1 daily for a period of 35 days. The second group of animals served as positive controls and, in consequence, each animal received 6 μg. of vitamin B_1 daily throughout the experimental period.

DATA

The data reported at this time have been reduced to group averages and are given in figures 1 to 5 inclusive. In making these tabulations only data from those animals which survived the entire experimental period have been included.

[2] Obtained from The Borden Company, Bainbridge, New York. Two hundred grams of this product were dissolved in 750 ml. of distilled water, autoclaved for 4 hours at 15 pounds pressure, cooled, filtered and made up to 1 liter.

DISCUSSION

It was observed that animals in the "forced exercise" group were unable to undergo the desired 1000 cage revolutions daily, during the first few days of the experiment, without becoming exhausted. As a result, the exercise requirement for these animals was less than 1000 revolutions per day, during this period. The number of revolutions, daily, was gradually increased until all animals were meeting the specified exercise requirement of 1000 revolutions per day, by the end of the first week.

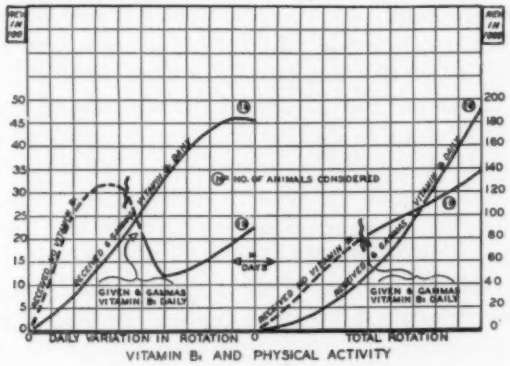
NO. OF ANIMALS CONSIDERED
GIVEN 6 GAMMAS VITAMIN B1 DAILY
GIVEN 6 GAMMAS VITAMIN B1 DAILY
DAILY VARIATION IN ROTATION
TOTAL ROTATION
VITAMIN B1 AND PHYSICAL ACTIVITY

Fig. 1 The mean variation in physical activity of two groups of young rats maintained under comparable conditions in all respects except as to their vitamin B_1 intake.

In the course of these studies a rather significant observation was made in that those animals of the voluntary exercise groups which were depleted of their vitamin B_1 reserve were found to be more active physically, during the first few weeks of the depletion period, than comparable animals, similarly caged, but receiving 6 μg. of vitamin B_1 daily (fig. 1). During the first day or so of the experiment all animals of the voluntary exercise group deported themselves similarly. By the end of the first week, however, it was noted that the animals which were not receiving the vitamin supplement were rotating the wheels of their respective cages more frequently than

were the animals which received the vitamin supplement. This condition became even more noticeable as the experiment progressed. By the twenty-first day of the experiment the animals undergoing depletion were making twice as many revolutions as litter mates receiving thiamin, and approximately three times as many revolutions as the animals of the forced exercise group. However, as the onset of the symptoms of vitamin B_1 deficiency became more pronounced, these animals became progressively less active as paralytic symptoms developed. When the animals reached the stage of depletion where physical excitation resulted in spastic paralysis, they were fed the vitamin supplement. In most instances, it was surprising to observe that such animals continued to become even less active for several days after the supplementary feeding was begun. Usually (about the fourth day of the supplementary feeding of 6 μg. of vitamin B_1 daily), these animals began to regain some of their inclination to exercise and continued to do so until the end of the experiment. However, at the end of the experiment, the average daily activity of the rejuvenated animals was considerably less than the daily activity of similar animals which had received the vitamin from the beginning of the experiment. A possible explanation for this may be found in the relationship of vitamin intake to the weight of the experimental animals. This observation would appear to bear some significance in any attempt to estimate the optimal requirement of the rat for vitamin B_1. In this regard, it appears from the data presented in figure 1, that an intake of 6 μg. of vitamin B_1 is insufficient to maintain the growing rat (50–120 gm.) in a state of nervous stability and that this inadequacy becomes even more pronounced as the weight of the rat increases. Therefore, if such is the case, it is to be expected that the smaller animals receiving 6 μg. of vitamin B_1 daily will be found to be more stable physically than larger animals receiving similar amounts of the vitamin. A further suggestion that the quantity of vitamin B_1 used in these studies (6 μg. daily) was not adequate to maintain the growing rat in a state of nervous equilibrium is found in the fact that the animals used in these studies were far more active physi-

cally than were the animals used in our previous studies where a basal diet containing 8% of dried yeast was fed.

An inspection of the data presented in figures 2 and 3 reveals that exercise, under the conditions in which it was employed in these studies, had a measurable effect on the growth rate of young rats irrespective of whether or not the animals were receiving the vitamin B_1 supplement. Those animals of the voluntary exercise group, which were most active

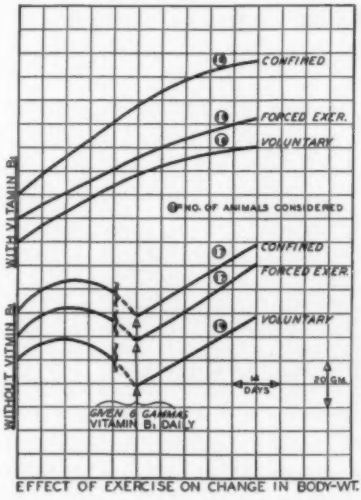

Fig. 2 The effect of different amounts of exercise on the growth rate of young rats while being maintained under comparable dietary conditions except as to their vitamin B_1 intake.

in rotating their cage wheels made smaller increases in body weight than did the less active animals. This observation was most striking during the depletion period, where the animals of the voluntary exercise group showed a smaller initial increase in body weight (8 gm.) and in turn lost a greater percentage of this weight (32%) before developing the paralytic symptoms. In fact the mean weight of the animals of this group at the end of the depletion period was 41 gm., or 11 gm. less than their initial weight (when placed on experiment). The maxi-

mum gains in weight for the three groups of animals (voluntary exercise, forced exercise, and confined) during the depletion period were 8, 13, and 14 gm. respectively, while the subsequent losses in weight for the same groups of animals were 20, 16, and 15 gm. respectively. The mean increase in body weight following the supplementary feeding of vitamin B_1 was found to be quite comparable for the three conditions of exercise. These values, when given in the above order of groups, were 29, 33, and 30 gm. respectively. In this connection, it should be borne in mind that the activity of the animals

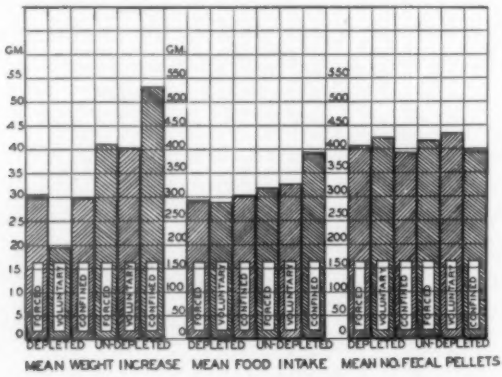

Fig. 3 The relative effects of exercise on the mean body weight increase, the mean food intake and the mean number of fecal pellets voided by young rats while receiving diets identical in all respects except in their vitamin B_1 content.

of the voluntary exercise group, during this time, was considerably less than during the depletion period, and probably more comparable to the activity of the forced exercise animals. In the instance of the animals which received the vitamin supplement from the beginning of the experiment, those of the confined group grew at a considerably faster rate than did the animals of either of the other two groups (voluntary and forced). While it is possible that the nature or the composition of the body weight increments produced under the different conditions of exercising may have been quite different, no data are at hand to substantiate this view. However, later studies

on the chemical composition of body tissues may shed further light on this problem.

From the standpoint of food consumption, the various degrees of exercise did not appear to be an important consideration (fig. 3). Although under both conditions of vitamin supplementation, the animals of the confined groups consumed the greatest amounts of food during the experimental period, the relationship of food intake to body activity is not clearly defined in all instances. It is evident from these data that the matter of growth is a complex phenomenon which is influenced by a number of factors, among which are food intake and exercise.

A similar consideration of the fecal output of the various groups of animals (fig. 3) reveals that there was no great variation in the number of fecal pellets voided by the respective groups of animals during the entire experimental period. However, it seems significant that in both instances the animals of the voluntary exercise groups voided the greatest number of fecal pellets while the animals of the confined group voided the smallest number of fecal pellets. A tabulation of the data regarding the effect of exercise on the weight of the fecal matter, the percentage of fecal matter (relative proportion of food excreted as fecal matter) and the relative sizes of the fecal particles (fig. 4) reveals some significant differences between the animals of the three experimental groups in these respects.

The animals of the confined groups produced the greatest weight of fecal matter, the greatest percentage of fecal matter and the largest fecal pellets (as indicated by weight), while the animals which exercised most strenuously (voluntary exercise group) produced the least fecal matter, the smallest percentage of fecal matter and the smallest fecal pellets. While some of these differences may not be significant, the data clearly indicate a beneficial effect of exercise on fecal elimination. Another interesting observation relates to the fact that under all conditions of exercise, those animals which received vitamin B_1 utilized their food more efficiently (lower

percentage of fecal matter) than did similar animals not receiving the vitamin supplement.

The data given in figure 5 demonstrate the effect of exercise on the average time required for the animals of the various

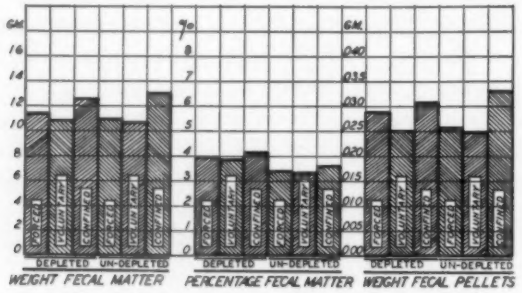

Fig. 4 The effect of exercise on total weight of fecal matter, percentage of fecal matter and mean weight of fecal pellets voided by young rats while receiving diets identical in all respects except as to the amount of the vitamin B_1 supplement.

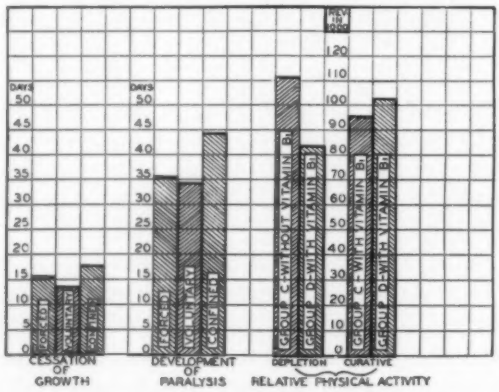

Fig. 5 The effect of exercise on the time required for the cessation of growth and for the development of the paralytic symptoms among groups of young rats receiving a diet deficient in vitamin B_1. The figure also shows the relative activity (as indicated by the rotation of the cage wheel) of two comparable groups of young rats, of which the animals of one group (group D) received daily 6 μg. of vitamin B_1 throughout the experimental period, while the animals of the other group (group C) received no added vitamin B_1 during the depletion period but received daily 6 μg. of this vitamin during the last 35 days of the experimental period.

groups, undergoing depletion, to cease growing. It will be noted that the animals of the voluntary exercise group ceased growing after about 13.5 days on the deficient diet, while the animals of the forced exercise and of the confined groups required 16 and 17.6 days, respectively. Corresponding data relative to the average time required for the development of the paralytic symptoms were equally significant. The animals of the voluntary exercise group manifested the characteristic paralytic symptoms on the thirty-fourth day of the depletion period, while the animals of the forced exercised and of the confined groups required 36 and 44 days respectively. These findings clearly indicate that young rats undergoing vigorous exercise have somewhat greater requirements for vitamin B_1 than do similar animals which undergo little or no exercise. This is in agreement with the data obtained by Cowgill and co-workers ('31) in their studies with dogs but is not in agreement with our previous findings regarding the influence of exercise on the vitamin A requirement of the growing rat. However, it seems highly probable that the latter variation may be due to differences in the physiological function of the two vitamins. It appears reasonable to postulate that the symptoms of vitamin A deficiency are accentuated by the accumulation of toxic substances in the body of the experimental animal due to poor elimination. Therefore, any factor, such as exercise, which increases elimination may bring about a delay in the development of such symptoms. On the other hand, vitamin B_1 which is known to play an important role in the complicated phenomena associated with cellular oxidation and muscle contraction may be required in increased amounts during periods of vigorous exercise. In fact the data obtained in these studies indicate that the vitamin B_1 requirement of the rat is more dependent on the rate of metabolism than it is on the caloric intake.

SUMMARY

Experiments conducted with carefully selected groups of young rats as experimental subjects have yielded data which demonstrate that the vitamin B_1 requirement of these animals,

as indicated by the time required for the cessation of growth and for the development of the paralytic symptoms, is increased by physical exercise. These findings are not in agreement with previous findings concerning the influence of exercise on the vitamin A requirement of the growing rat. A possible explanation of this variation may be found in the differences in the physiological function or functions of the two vitamins.

In the instance of the animals receiving no vitamin B_1, increased exercise resulted in less food consumption, less growth, early development of paralytic symptoms and the elimination of a greater number of fecal particles. With the animals receiving 6 μg. of vitamin B_1 daily, increased exercise resulted in less growth and in the elimination of a greater number of fecal pellets. During the first 4 weeks of the test period, those animals which were permitted to exercise voluntarily were more active physically while not receiving vitamin B_1 than were comparable animals, similarly caged, but receiving vitamin B_1. However, during the latter stages of vitamin B_1 depletion, physical activity among these animals declined to a minimum of about one-third of its previous value. This decline in physical activity continued for approximately 6 days after the administration of daily dosages of 6 μg. of vitamin B_1. There was a steady resumption of activity by these animals during the last 4 weeks of the experimental period, as the result of the administration of the vitamin. Definite nervous instability resulting from vitamin B_1 deficiency was indicated, during the early part of the depletion period, by the increased tendency of the animals to exercise.

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STUDIES ON A DERMATITIS IN CHICKS DISTINCT FROM PANTOTHENIC ACID DEFICIENCY [1]

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(Received for publication August 12, 1940)

ONE FIGURE

Extensive work in several laboratories (Woolley et al., '38, '39; Jukes, '39; Williams and Major, '40) has established the essential nature and role of pantothenic acid in the prevention and cure of chick dermatitis, and generally "chick dermatitis" is used to refer to pantothenic acid deficiency. However, it has been known for several years that similar lesions may be produced in chicks and other animals by the inclusion of raw egg white in the ration. Lease and Parsons ('34) and others demonstrated that the chick antidermatitis factor was not curative for this condition and further studies have supported this conclusion. Although rats have been used to a large extent in the investigations upon the anti-egg white injury factor, termed vitamin H by Gyorgy ('37), there is no reason to believe that the results obtained do not apply to chicks. The properties, distribution, and concentration of

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We are indebted to Merck and Company, Rahway, New Jersey, for generous supplies of thiamin and vitamin B_6; to the Abbott Laboratories, North Chicago, for haliver oil; to the Pabst Brewing Company, Milwaukee, for yeast; to Wilson Laboratories, Chicago, for liver extract; and to Allied Mills Inc., Peoria, Illinois, for soybean oil.

vitamin H have been investigated most extensively by Gyorgy and associates (Gyorgy, '39; Birch and Gyorgy, '39; Gyorgy, Kuhn and Lederer, '39). Recently Gyorgy, Melville, Burk and du Vigneaud ('40) have pointed to the marked similarity of vitamin H and biotin, the bacterial growth factor, and state that no serious difficulty is encountered in correlating the distribution and properties of these factors. Potent concentrates of vitamin H were also said to be high in biotin.

During studies on purified rations for chicks in this laboratory we have observed many cases of dermatitis upon rations apparently adequate in pantothenic acid. In this paper we wish to report studies on this condition. The preventive and curative factor is similar to vitamin H and biotin in distribution and properties and potent vitamin H concentrates have also been found to be active.

EXPERIMENTAL

The rations used in these studies have varied in the kind and amount of liver extract used. Essentially they had the following percentage composition: purified casein 18, salt mixture 5, defatted cartilage 15, soybean oil 5, liver extract (fraction D)[2] 3 to 8, and dextrin 49 to 54. Thiamin and vitamin B_6 were added at the rate of 2 mg. per kilo and a vitamin A and D concentrate was fed separately. In some cases a yeast eluate was added to supply a growth factor necessary in addition to alcoholic liver extracts (Hegsted et al., '40 a) which is presumably factor U (Stokstad and Manning, '38). The liver extracts used have been shown to supply adequate pantothenic acid and flavin at a 2% level and cartilage supplies the cartilage growth factor (Hegsted et al., '40 b).

Day-old White Leghorn chicks were used throughout. They were placed on raised screens in small heated brooders with the experimental ration and water supplied ad libitum. Weighings were made weekly and the chicks were examined frequently for lesions on the feet and around the beak.

[2] Wilson Laboratories.

RESULTS

Although some variation has been found in the time necessary for the depletion of different groups of chicks, incipient lesions similar to those seen in egg white toxicity usually appear in about 3 weeks. The bottoms of the feet become rough and calloused and may be severely affected before mandibular lesions are evident. As the syndrome progressed the entire bottom of the foot becomes encrusted and hemorrhagic cracks appear (fig. 1). The toes may become necrotic and slough off

Fig. 1 Dermatitis produced on a ration adequate in pantothenic acid. Note the severity of the foot lesions.

but the top of the foot and leg usually show only a dry scaliness. The mandibular lesions which first appear in the corners of the mouth spread to include the area around the beak, and the eyelids become swollen and stick together. In contradistinction to these symptoms the lesions in pantothenic acid deficiency are first evident in the corners of the mouth and seldom if ever do the lesions on the feet become as severe as in the new syndrome described.

The preventive effect of various supplements is shown in table 1. The average weight at 4 weeks of age is also given although antidermatitis potency is not paralleled by growth effect upon this ration. When 8% of liver extract is included in the ration large amounts of pantothenic acid are supplied.

TABLE 1

The activity of various supplements in the prevention of dermatitis

SUPPLEMENT TO BASAL RATION	NUMBER OF CHICKS	AVERAGE WEIGHT AT 4 WEEKS	NUMBER DEAD AT 4 WEEKS	DERMATITIS		
				None	Slight	Severe
		gm.				
None	29	132	5[1]		5	21
2 mg. pantothenic acid per 100 gm.	6	128	1			5
5% commercial yeast extract	6	138				6
5% Vitab	11	152		1	2	8
10% Vitab	6	137			3	3
3% molasses	6	137				6
6% molasses	6	130			4	2
12% molasses	6	142		2	3	1
20% heated middlings	5	161	1	1		3
45% heated yellow corn	11	152		4	6	1
10% fishmeal (replacing casein)	6	159		1	2	3
49% polished rice	6	177		2	2	2
2% kidney residue	6	178		6		
3% kidney residue	12	159	2	10		
6% kidney residue	6	170		6		
MeOH ext. of papain digest of kidney residue = 5%	6	132	1	5		
3% liver residue	6	153		6		
5% brewers' yeast	11	182	1	10		
5% heated brewers' yeast	6	169		5	1	
10% yeast residue	11	171	3	8		
2% kidney residue + 15% additional cartilage	6	172		6		
22% additional casein (no cartilage)	6	96			5	1

[1] Two of these died late enough in the 4-week period to be examined for dermatitis.

However, to rule out definitely its effect the ration was further supplemented with a pantothenic acid concentrate. No growth response or prevention of dermatitis was observed. Yeast extract, a commercial rice polish concentrate,[3] and molasses were relatively poor sources of the antidermatitis factor.

[3] Vitab.

Heated middlings and yellow corn, fish meal and polished rice were only partially effective at rather high levels. Complete protection was obtained with 2% of kidney residue prepared by extracting dried defatted kidney with large volumes of hot water and 50% alcohol. The factor is obviously very difficult to extract from kidney but proved to be water and methanol soluble after digestion of the kidney residue with papain. Liver residue and brewers' yeast were also potent sources of the factor. Five per cent of brewers' yeast heated to destroy pantothenic acid allowed only one slight case, and yeast residue after 50% methanol extraction was also effective.

In order to eliminate the possibility that cartilage was acting in a manner similar to egg white, a low level of kidney residue was added together with additional cartilage, but no dermatitis occurred. Also, in one group the cartilage was completely eliminated and replaced by casein. Growth was poor but all of the chicks developed dermatitis which was later cured by kidney residue.

Curative tests have also been used successfully. Since spontaneous cures have not been noted this procedure may be more satisfactory than the preventive method. The protocols (table 2) show that beef spleen and round were less potent than either kidney or yeast. Considerable variation may be noted and 10% of these materials produced only slight improvement in 3 weeks in two of the chicks. Potent sources, such as 6% of brewers' yeast, or kidney, or liver residue, produced dramatic responses; severely affected chicks were cured in less than 2 weeks. A water soluble preparation prepared by autoclaving liver residue for 1 hour with 15% sulfuric acid was also active.

Potent preparations of vitamin H were supplied by Dr. du Vigneaud.[4] Preparation II contained 200 rat units of vitamin H (Gyorgy, '39) per milligram while preparation III was less pure and contained 25 units per milligram. These were injected twice weekly to supply the levels indicated in table 2. As can be seen, the highest level produced complete cures in 3 weeks and lower levels were nearly as effective.

[4] We wish to express our appreciation to Dr. V. du Vigneaud, Cornell Medical School, New York, for sending us the concentrates of vitamin H.

TABLE 2

Curative effect of various supplements

SUPPLEMENT TO BASAL RATION	CHICK NO.	AGE AT START	DERMATITIS SCORE AT [1]		
			1 week	2 weeks	3 weeks
		weeks			
None	9844	4	3	3	3
None	9845	4	3	3	3
None	9846*	4	2	3	3
5% dried beef spleen	547	5	3	3	1
5% dried beef spleen	537	5	2	1	1
10% dried beef spleen	539*	5	2	1	?
10% dried beef spleen	545	5	3	3	2
5% dried beef round	541	5	2	1	?
5% dried beef round	546*	5	2	1	1
10% dried beef round	540	5	3	3	2
10% dried beef round	550	5	3	3	1
6% liver residue	551	6	3	?	
6% liver residue	560	6	3	0	
15% H_2SO_4 ext. = 5% liver residue	617	4	3	2	?
15% H_2SO_4 ext. = 5% liver residue	603	4	3	?	0
3% brewers' yeast I	544	5	3	2	1
3% brewers' yeast I	552	5	3	3	1
3% brewers' yeast II	558	5	3	2	1
6% brewers' yeast I	549*	5	1	0	0
6% brewers' yeast I	555	5	2	1	0
3% kidney residue	843	4	2	1	0
3% kidney residue	847	4	3	2	0
3% kidney residue	848	4	3	2	?
6% kidney residue	559	3	2	0	
MeOH ext. of papain digest = 1 gm. kidney/week, injected	842	4	2	0	
(Prep. II) 200 units of vitamin H/ week, injected [2]	601	4	3	?	0
(Prep. III) 200 units of vitamin H/ week, injected	607	4	2	?	0
(Prep. II) 100 units of vitamin H/ week, injected	602	4	3	2	?
(Prep. III) 100 units of vitamin H/ week, injected	611	4	3	1	0
(Prep. II) 50 units of vitamin H/ week, injected	610	4	2	?	0
(Prep. III) 50 units of vitamin H/ week, injected	608	4	3	1	?

[1] Chicks marked with asterisk had a dermatitis score of 2 at the start; all others scored 3. A dermatitis score of 3 indicates severe dermatitis on both feet and beak. Smaller numbers indicate less severe cases. Doubtful cases are marked by ? and 0 indicates complete freedom from lesions.

[2] Preparations II and III were assayed for biotin by the bacteriological method by J. O. Lampen and W. H. Peterson. Preparation II contained 5 μg. per milligram and preparation III 0.56 μg. per milligram.

DISCUSSION

The inactivity of liver extract or pantothenic acid preparations, the severity of the foot lesions, and the properties of the active factor clearly differentiate this condition from chick dermatitis caused by lack of pantothenic acid. On the other hand, the similarity of the factor to biotin and vitamin H is indicated by distribution, resistance to extraction, liberation by papain or acid hydrolysis, and stability to heat and strong acid hydrolysis. Finally the activity of potent vitamin H concentrates suggests the similarity of these factors. It should be emphasized that chicks have been cured in 3 weeks by injection of approximately 35 μg. of solids per day. This is a much higher level than rats require but we have no indication that this is a minimum level. Also our chicks weigh from 150 to 250 gm. and we have used a 3-week curative period whereas a 4-week period is used in the rat assay (Gyorgy, '39). Lease ('37) has indicated that chicks require higher levels of vitamin H than rats on rations containing egg white. Thus it may not be surprising that chicks require higher levels than rats, and this may explain why no deficiency of vitamin H has been observed in rats unless egg white is included even though the rations are more highly purified than those we are using for chicks.

It is probable that upon our ration a true deficiency exists since raising the level of cartilage apparently does not increase the requirement and the deficiency has been produced with casein as the sole source of protein. However the possibility remains that all proteins may have the effect of egg white to some extent and that this would only be evident when purified rations low in the protective factor are used.

Although fishmeal, polished rice, middlings, etc., are not potent sources of the factor, they probably contain a sufficient amount of it to explain the failure of other investigators to encounter this condition. Vitamin supplements prepared by autolysis or hydrolysis of liver or yeast may also carry the active factor.

SUMMARY

A typical dermatitis occurring in chicks fed purified rations adequate in pantothenic acid has been described. The properties of the protective factor agree with those reported for vitamin H and biotin and potent preparations of vitamin H have produced cures in 3 weeks when injected at a level of 35 μg. per day, the lowest level used.

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STUDIES ON THE THIAMIN REQUIREMENT OF YOUNG SWINE

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ONE TEXT FIGURE AND ONE PLATE (FOUR FIGURES)

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The present investigation on the thiamin (vitamin B_1) requirement of swine was suggested from observations on the association of dietary differences with growth and mortality of suckling pigs. Experimental work (unpublished) has demonstrated the value of skim milk as a supplemental feed to the regular ration of corn, mineral mixture and trinity mixture of tankage, 50 parts; alfalfa leaf meal, 25 parts; and linseed meal, 25 parts in the "creep" feeding of unweaned pigs. Losses from birth to weaning from various causes frequently amount to one-third of the total number of pigs farrowed. In addition, there is a considerable loss due to stunted growth and uneconomical gains. While most swine rations appear to contain liberal amounts of thiamin, the high requirement of the lactating female in general for this factor raises the question as to the nutritive state of the growing young. Accordingly, attention has been focused on pigs between the ages of 3 and 12 weeks when considerable food is required in addition to the dam's milk.

Since the present work was initiated, a number of reports, including those of Hughes ('38, '39), Chick and co-workers ('38), Wintrobe, Mitchell and Kolb ('38), Wintrobe ('39), and Davis, Freeman and Madsen ('40) have appeared on the role

of various members of the so-called vitamin B complex in the nutrition of swine. It now appears reasonably certain that thiamin, riboflavin, nicotinic acid, and perhaps several more additional factors are required.

One of the major problems in experimental studies on the requirements of swine for components of the vitamin B complex is that of a suitable basal diet. It is necessary that the diet be readily and economically prepared in quantity to enable the feeding of growing pigs for extended periods. While methods for study of thiamin appeared to be better established than for other factors, their application to the feeding of young pigs has remained in doubt.

The work to date has dealt with the response of pigs fed diets in which the thiamin was either destroyed by autoclaving or by sodium sulfite-sulfur dioxide treatment. A requirement for thiamin by young pigs has been demonstrated and a growth response and alleviation of a number of symptoms have followed supplementary feeding of graded amounts of crystalline thiamin hydrochloride to animals maintained on these deficient diets. Some of the physiological effects which have been encountered are similar to those described by the investigators already mentioned. Certain difficulties have been encountered in the ability to restore to and to maintain an optimum state of well-being by the feeding of thiamin along with rations treated to free them of vitamin B_1 by autoclaving. There has appeared to be a marked difference in the results obtained with the autoclaved diets and with the sulfite-sulfur dioxide treated diets, due perhaps to differences in destruction or inactivation of one or more other essential factors. In addition, other information on pathological changes in the organs and tissues has been gained which emphasizes the complexity of the problem.

EXPERIMENTAL PROCEDURE

The experimental work was conducted at the Beltsville Research Center, Beltsville, Maryland, between May, 1938 and February, 1940. In the preliminary experiments, the constitu-

ents of the diets which contained factors of the vitamin B complex were autoclaved in the usual manner and in the main series the same constituents were treated with sodium sulfite and sulfur dioxide to destroy thiamin according to the method described by Kline, Tolle and Nelson ('38). Besides the pig feeding work, parallel feeding experiments were conducted with rats which in a general way supported the findings on the pigs, although the symptoms seen in the swine are not comparable in all details to those observed in rats.

Pigs were obtained from the Bureau of Animal Industry herd at Beltsville, Maryland, at the age of 3 weeks and before they had begun to eat much feed other than the dam's milk. Not more than seven pigs were on experiment at any one time and each group represented a selection from either one or two litters. Most of the pigs were crossbred Poland China and Landrace while a few were purebred Poland China. The total number used during the 2 years was thirty-six. They were housed individually in metabolism cages on ½ inch screen floors in a room kept at 75° ± 2° F.

The composition of the sulfite-sulfur dioxide treated diets used in the main experiments is given in table 1. Alcohol- and acid-extracted casein was used throughout except for the control pig on diet CS5 in which case the casein was not treated. In diet CS7 untreated whey was used as a source of thiamin except for pig 2952 in which case additional pure thiamin was given. In diet CS5 the untreated whey and casein supplied the thiamin. It will be noted that the liver and mineral contents of diet S7 were increased over that of diet S5. The autoclaved diets were of similar composition to those in table 1. The liver and whey were autoclaved and dried in a vacuum shelf dryer. The lard was included at a level of 10% as representing an approximation to the fat intake of pigs being nursed by their dams but consuming considerable additional grain feed.

The animals were fed four times in each 24 hours for the first 3 weeks on experiment and thereafter three times a day. The basal diets were fed as a thin slop. The pigs were gener-

ally given a daily allowance of feed equivalent in weight to 4% of their body weight in terms of air dried feed, as long as their appetites were normal and thereafter lesser but regulated amounts suited to the rate of consumption. Effort was made to secure a uniform and regulated feed intake which could be recorded and compared with the thiamin intake and to the rates of growth. The fortified cod liver oil was listed as

TABLE 1

Percentage composition of the sulfite-sulfur dioxide treated diets

CONSTITUENTS	DIET NUMBER [1]		
	S5	S6	S7
Dried liver, SO_2 treated	5.0	8.0	7.5
Dried whey	15.0	15.0	15.0
Casein	25.0	25.0	25.0
Dextrin	20.0	20.0	27.5
Sucrose	21.0	18.0	9.5
Lard	10.0	10.0	10.0
Agar	2.0	2.0	2.0
Fortified cod liver oil	0.2	0.2	..[2]
Salt mixture	1.8	1.8	3.0
Calcium carbonate			0.5

[1] In the deficient diets, designated with the prefix D, the whey was treated with sulfite-sulfur dioxide and the casein extracted with water and alcohol. In the control diets designated with the prefix C, the whey was left untreated. The sulfite-sulfur dioxide treatment was applied for 8 days (in diets DS7 and CS7) instead of the usual 5-day period except in the case of pigs 3364 and 3366.

[2] In diet S7 the cod liver oil was fed at the level of 0.75 cc. per kilogram body weight per week.

containing 3000 U.S.P. units of vitamin A and 400 A.O.A.C. chick units of vitamin D per gram. Crystalline thiamin chloride hydrochloride abbreviated subsequently to "thiamin" dissolved in water was fed at different levels on a per-kilogram-body weight basis. It was added to the feed in the autoclaved series and fed by mouth three times per week in the sulfite series.

RESULTS

Experiments with pigs

In order to use nursling pigs in the feeding of diets composed largely of purified food substances, it was necessary

to determine whether normal health and growth could be maintained on control diets in which the thiamin and the other factors of the vitamin B complex were supplied in their natural state. In addition it was necessary to determine if the pigs could be reared in pens or cages equipped with screen floors. Control animals apparently met these requirements in a reasonably satisfactory manner. The pigs gained in weight, maintained their appetites and were essentially free of those symptoms and abnormalities which characterized the pigs fed treated diets and given either no or low levels of thiamin. The first twelve pigs used in the preliminary experiments with autoclaved materials suffered from other deficiencies than thiamin since, when ample thiamin was added, the animals did not grow at a normal rate and most of them developed convulsions and paralysis of the limbs accompanied by severe myelin degeneration of the cord and sciatic nerve. In two cases, liver concentrate appeared to be effective in curing or preventing these pathological symptoms. The animals also had severe scours and in some cases voided very dark colored urine. The convulsions were similar if not identical to the fits in swine described by Chick, Macree, Martin and Martin ('38) when their animals did not receive a chemically unidentified member (or members) of the vitamin B complex. The nerve degeneration observed was similar to that found by Ellis and Madsen ('40) in swine which developed incoordination when fed on certain natural feed combinations and heated diets and also to that described by Wintrobe, Mitchell and Kolb ('38). Since a thiamin deficiency uncomplicated by other deficiencies could not be obtained by autoclaving the vitamin B-carrying components of the diet, the change to sulfite-sulfur dioxide treatment was made.

Data on the young pigs used in the experiments with sulfite-sulfur dioxide treated diets are given in table 2 and in figure 1. Five animals received the deficient diets without regular feedings of thiamin and a sixth received such a small amount, namely, 6 μg. per kilogram body weight, as to be ineffective in prolonging the life of the animal. The histories of these

TABLE 2

Results on pigs fed diets treated with sulfite and sulfur dioxide

THIAMIN FED		PIG NO.	DIET NO.	DAYS ON EXPT.[1]	WEEKLY GAIN [2]	APPETITE	VOMITING	NEAR DEATH OR END EXPT.		AUTOPSY		
Per kg. body wt.	Day started							Leg deformity	Rectal temp.	Heart	Alimentary tract	Body condition
µg.					*kg.*				*0°F.*			
0	—	2031	DS5	D. 28	Loss	Poor	Some	None	——	Flabby	Erosions	Emac.
0	—	2586	DS5	M. 33	Loss	Poor	Frequent	None	99.4	Flabby	Normal	Emac.
Two doses[4]	28–58	2705	DS5	M. 133	Varied	Varied	Some	None	93.5	Flabby	Normal	Emac.
0	—	2957	DS7	D. 34	Loss	Poor	Frequent	None	96.0	Pale	Congested	Emac.
0	—	3368	DS7	D. 39	Loss	Poor	Frequent	None	99.3	Mottled	Congested	Emac.
6	21	2958	DS7	D. 34	Loss	Poor	Frequent	None	100.3	Normal	Congested	Thin
15	15	3364	DS7	D. 36	2.1	Fair	Some	None	102.8	Pale	Normal	Good
15	15	3363	DS7	E. 85	1.4	Fair	None	Slight	102.4	Flabby	Normal	Fair
25	1	2032	DS5	E. 94	2.3	Good	None	Marked	——	Normal	Normal	Good
25	1	2587	DS5	E. 113	2.7	Good	None	Moderate	103.0	Normal	Normal	Good
25	15	3366	DS7	E. 85	2.5	Good	None	Moderate	103.2	Normal	Normal	Good
25	15	3365	DS7	E. 85	2.2	Good	None	Slight	102.6	Normal	Normal	Good
12 to 38	18	2959	DS7	E. 125	3.4	Varied	None	None	102.8	Normal	Normal	Good
18 to 38	18	2960	DS7	E. 125	3.3	Varied	None	None	103.8	Normal	Normal	Good
25 to 50	18	2951	DS7	E. 97	2.6	Varied	Slight	None	102.3	Normal	Normal	Fair
50	1	2033	DS5	E. 94	2.3	Good	None	Marked	——	Normal	Normal	Good
50	1	2706	DS5	E. 99	2.4	Good	None	Moderate	102.7	Normal	Normal	Good
50	1	2062	DS6	E. 94	2.5	Good	None	Marked	——	Normal	Normal	Good
50	15	3367	DS7	E. 85	3.4	Good	None	Moderate	103.4	Normal	Normal	Good
100	1	2034	DS5	D. 80	2.3	Good	None	Slight	——	Normal	Congested	Fair
100	1	2707	DS5	E. 99	1.1	——	None	Severe	103.4	Normal	Normal	Thin
Control	—	2035	CS5	E. 94	4.2	Excellent	None	None	——	Normal	Normal	Good
Control	—	2952	CS7	E. 97	2.1[3]	Good	None	None	102.8	Normal	Normal	Good
Control	—	3362	CS7	D. 41	1.9	Good	None	None	100.4	Normal	Congested	Good

[1] D. indicates death; M. moribund condition and E. that animal survived the experiment in fair or normal condition.

[2] In the case of the animals fed thiamin, the weekly gain is calculated for the period during which a designated level was fed. For animals 2951, 2959, and 2960 the gains for the last 28 days during which the maximum levels of thiamin were fed were used in the calculation.

[3] Food allowance was limited to a 3% ration for first half of the experiment.

[4] Each dose contained 1.8 mg.

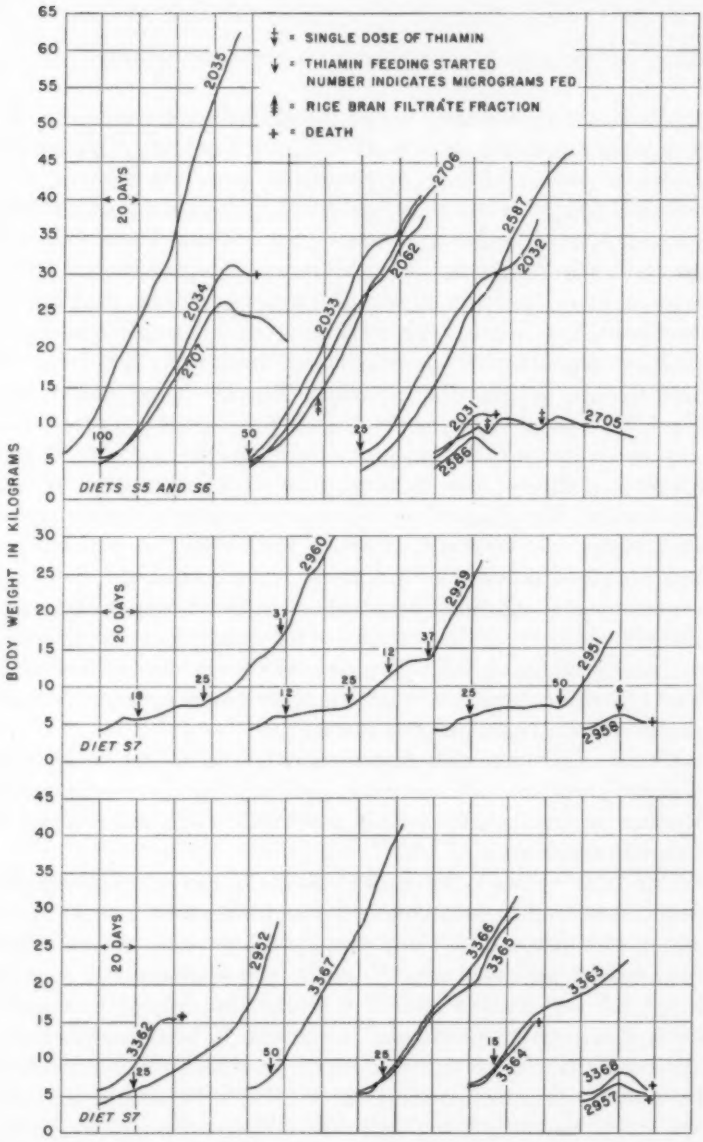

Fig. 1 Growth of pigs fed diets of the sulfite-sulfur dioxide series. The level of thiamin fed per kilogram body weight is indicated by the number (in micrograms) over the arrows pointing downward. Pigs 2035, 2952, and 3362 were positive controls. The remainder were on the deficient diets. Pig 2952 was restricted in food intake during the first part of the experiment.

pigs were characterized, as in the autoclaved series, by decreases in appetite and in body weight after the initial 3-week depletion period. Frequent vomitings were also noted.

Rectal temperature readings showed a marked decline from normal for several days preceding death. Four animals showed congested areas in the stomach or intestines. These same animals gave evidence of cyanosis at the time of or shortly before death. Pig 2031 died unexpectedly and upon autopsy considerable straw-colored fluid was found in the thoracic and pericardial cavities. Besides being flabby, the heart was paler than normal and the cardiac muscle was streaked with gray tissue particularly in the wall of the left ventricle. There was considerable serous atrophy of the cardiac fat. Histological examination revealed scattered areas of atrophy and necrosis of heart muscle fibers, a condition suggestive of infarction. On gross examination the liver appeared congested. Sections of liver tissue showed widespread central necrosis of the lobules with congestion around the periphery of the lobule. The lesions of the alimentary tract noted in table 2 were confined largely to the gastric mucosa in the pyloric region of the stomach.

Degenerative changes were found in the cardiac muscle of the walls of the left ventricle and of the inter-ventricular septum and in the liver of pig 2586 which was killed when in a moribund condition.

Pig 2705 showed the usual decline in weight during the fourth week on experiment and was then given a total of 1.8 mg. of thiamin over a 4-day period. There was an immediate response in increased appetite, gain in weight and rise in body temperature. By the end of 8 weeks the animal was again listless, without appetite and subnormal in body temperature. A second dose of 1.8 mg. of thiamin produced results identical to those in the previous instance. The body weight increase was approximately 2 kg. each time and was reached in 7 days. Following this final period of weight increase, the animal was kept on experiment for 6 weeks longer. The body temperature declined to 93.5° F. On the one hundred and thirty-third day

of the experiment, the animal was in a moribund condition and was killed. Extreme emaciation was the outstanding characteristic of the autopsy findings. As in several other cases, the heart appeared flabby. Histological examination of the heart muscle showed degenerative changes. Unlike other pigs, which received no regular thiamin therapy, the alimentary tract was apparently normal on gross examination.

Autopsy examinations on pigs 2957, 2958, and 3368 gave results similar to those just discussed. The changes in the heart tissues of these animals were especially noteworthy. The average time of survival on the thiamin-deficient diets for the five pigs was 35 days.

The feeding of thiamin gave favorable responses in the usual case. From a level of 15 to 50 μg. per kilogram body weight per day, there was evidence of a general improvement in rate of growth, appetite, and freedom from those abnormalities in organs and tissues noted among the animals on the deficient diets. On diets DS5 and DS6, good growth was obtained for 6 to 8 weeks on 25 μg. just as on 50 and 100 μg. of thiamin when no depletion period was allowed. Later, however, lameness developed among the animals. The symptoms resembled those of rachitic animals in contrast to the locomotor incoordination among pigs fed the autoclaved diets. Undoubtedly, confinement on the screen floors may have accentuated the stiffness and awkwardness in movement. Several of the animals receiving thiamin showed decreased appetite and rate of growth along with development of lameness. However, no convulsions developed, likewise in contrast to the results on the autoclaved diets. At one time during the feeding period all of the pigs of the 2032–2062 series developed acute diarrhea. Pig 2034 died presumably as a result of the attack. The chief findings at autopsy were congestion of the lungs and occasional areas of congestion in the small intestine. The diarrhea cleared up in the other animals in about a week. The difficulties with lameness and other evidence of dietary deficiencies among the animals just described prompted the feeding of a filtrate fraction of rice bran to pig 2706 from the

thirty-seventh to the seventieth day of the feeding period. As shown in table 2 and figure 1, this animal remained in good health except for a moderate degree of leg deformity. However, its condition was no different from that of other animals which did not receive the rice bran supplement, as for example no. 2587. Pig 2707 became very lame and was unable to stand upon its feet, even to eat and drink.

All the surviving animals fed on diets DS5 and DS6 were slaughtered after they had been on their experimental diets for 14 to 16 weeks, and no abnormal pathology was seen in the organs or intestinal tract. However, these animals as a group tended to have enlarged leg joints; bones appeared slightly softer than usual and the spinal column of some of the animals appeared more curved than normal. The serum calcium of the pigs on the treated diets was uniformly low and the inorganic phosphorus and the phosphatase values high. No abnormalities were noted in blood sugar, non-protein nitrogen, hematocrit or red and white blood cell counts. Bone analyses indicated an abnormally low ash content for the deficient pigs. These findings suggest a possible interference with mineral metabolism in the animals fed diets DS5 and DS6. A histological examination of the sciatic nerve and spinal cord of these animals also revealed some changes which indicate that the diets were probably deficient in other factors besides thiamin which was specifically studied in this series of experiments.

The pigs fed diets DS5 and DS6 showed a satisfactory response to thiamin treatment except for the rachitic-like lameness symptoms apparently resulting from deranged mineral metabolism and to a slight extent from mild nerve degeneration. Since calcium, phosphorus, and cod liver oil levels were suspected as playing a part, these nutrients were increased in diet S7 which was fed to the rest of the pigs. The cod liver oil was fed separately at the level of 0.75 cc. per kilogram of body weight per week. Although diets DS5 and DS6 were found to contain approximately 0.42% calcium and 0.56% phosphorus which is within recognized requirement standards, the calcium content was doubled and the phosphorus content

increased by 20% in the new diet. Another modification introduced in diet DS7 for the feeding of a number of pigs was the 8-day sulfite-sulfur dioxide treatment of the liver and whey. This was done because of the suggestion from both rat and pig feeding experiments that a 5-day treatment was insufficient to destroy all the thiamin. Pigs numbered from 2951 and above were fed the S7 diet.

The earlier work suggested that 25 μg. of thiamin daily per kilogram of body weight was possibly adequate for growth. Accordingly, thiamin was fed at 6, 13, 19, and 25 μg. levels, starting after 21 days on the deficient diet alone (fig. 1). Feed intake was restricted at first to 3% of body weight with some animals refusing to eat even this allowance. All levels of thiamin proved too low for promotion of normal growth. As indicated in figure 1, changes were made in the thiamin levels of pigs 2951, 2952, and 2960. It was found that a 37 μg. level resulted in better growth than 25 μg. and essentially as good as 50 μg. These three animals, like no. 2952 on the control diet, remained free of abnormal symptoms except for instances of loss of appetite, vomiting, and low body temperature alleviated by increased thiamin dosage.

The final group of seven pigs, all from one litter and numbered from 3362 to 3368, confirmed the results already obtained. Pigs 3365 and 3366 were fed 25 μg. of thiamin and gained 2.2 and 2.5 kg. per week respectively, as compared to pig 3367 which gained 3.4 kg. per week on 50 μg. of thiamin. Pigs 3362 and 3364 died before the conclusion of the test. Autopsy examination of pig 3362 fed diet CS7 showed considerable congestion in the intestinal tract, and, like pig 3364, which was given the 5-day treated diet and 15 μg. of thiamin showed an accumulation of free fluid in the body cavities. Other than these findings together with some diarrhea preceding death, the exact causes of death could not be determined. Unlike the pigs fed on diets DS5 and DS6, those on diet DS7 showed normal calcium, phosphorus and phosphatase values. However, locomotor abnormalities persisted to a slight degree.

Experiments with rats

Some results from rat feeding trials on the same diets as fed to the pigs are shown in table 3. The average weekly weight changes calculated for the last 5 weeks of the 7-week test period and the animal survival figures generally confirm the results obtained on the pigs. The rats which received no thiamin usually developed symptoms of polyneuritis. They were left on the test diet in the usual case until death. Rats on similar autoclaved diets did not grow as rapidly as those shown in table 3 when thiamin was fed as a supplement.

TABLE 3

Results on male rats fed the deficient diets during a 7-week period with and without added thiamin during the last 5 weeks

DIET	NO THIAMIN			60 μg. THIAMIN WEEKLY	
	Number of rats	Number alive after 7 weeks	Weekly loss in weight [1]	Number of rats	Weekly gain in weight [1]
			gm.		*gm.*
DS5	7	7	—1.1	6	18.7
DS7 (5 day)	7	3	—2.1	6	24.4
DS7 (8 day)	7	3	—4.2	6	23.3

[1] Figures based on changes in weight from the third to the seventh week inclusive.

The difference between the growth rate of rats on diet DS5 as compared with DS7 when thiamin was fed as a supplement was probably due to the increased percentage of liver in the latter diet. Treatment of the whey and liver for 8 days with sulfite and sulfur dioxide as compared to 5-day treatment was reflected in the weekly weight changes of the rats receiving no supplemental thiamin. More recent work (unpublished data) indicates however that with careful regulation of the pH of the sulfite-sulfur dioxide treating medium as complete a destruction of thiamin occurs when treated 5 days as when treated 8 days. It is recognized that small and probably variable amounts of thiamin may have been present in the basal diets which were not detected either in the pig or rat feeding work.

DISCUSSION

When whey and liver were autoclaved and vacuum dried, destruction of other vitamins than thiamin undoubtedly oc-

curred. On the other hand the treatment of whey and liver with sulfite and sulfur dioxide especially as used in diet DS7 seemed to be specific for the destruction of thiamin since the addition of thiamin alone enabled the pigs to grow at approximately a normal rate without developing other complicating deficiency symptoms to any marked extent.

The symptoms observed in the pigs on the autoclaved diets are worthy of note as they were probably caused by the destruction of other unknown factors necessary for the adequate nutrition of the pig. The myelin degeneration seen in the pigs on these experimental diets appears to have been due to other deficiencies than thiamin. This view is in accord with results obtained by Eveleth and Biester ('37), Wintrobe, Mitchell and Kolb ('38) and Ellis and Madsen ('40), and by other investigators using other animals than the pig.

The results obtained on diet DS5 and DS6 were complicated by inadequate mineral nutrition. This defect was largely corrected in diet DS7. It was of interest to note that pig 2035 on diet CS5 did not become lame nor was his serum calcium low. This would indicate that possibly the sulfite-sulfur dioxide treatment of the whey in some manner rendered the minerals of the whey unavailable to the pig.

An approximation of the vitamin B_1 requirement of young pigs appears to be indicated especially from the results obtained with animals on diet DS7. Apparently over the period of growth studied in these pigs the requirement per kilogram body weight is more than 25 μg. per kilogram per day, while a level of 37 and 50 μg. appears to be enough thiamin to maintain an approximately normal rate of growth. Cowgill's formula ('34) when applied to these small nursling pigs gives values much lower than the values obtained above; in applying his formula the maximum weight of the species was taken to be 380 kg. (see Russell, '22).

Although the animals were fed thiamin on a body-weight basis, the regulation of feed intake to the same basis and the keeping of approximate feed consumption records also permit the expression of thiamin intake on the basis of total feed

and of the sum of protein and carbohydrate. Thus a 4% feed allowance provides 40 gm. per kilogram body weight daily; and where the thiamin allowance was 37 μg. the thiamin intake per 100 gm. of feed was approximately 94 μg. Tabulation of actual data is given in table 4 for those pigs successfully reared on diet DS7. As would be expected the data in column 6 show an increase in thiamin intake per 100 gm. of feed as the thiamin allowance per kilogram body weight increased. It is interesting to note that in the first four cases where the thiamin intake ranged from 47 to 70 μg. the pigs actually maintained their body weight or even gained.

TABLE 4

Approximate thiamin intake per 100 gm. of feed consumed for pigs on diet DS7

PIG NO.	THIAMIN LEVEL PER KILOGRAM BODY WT.	DAYS FED ON GIVEN THIAMIN LEVEL	TOTAL APPROXIMATE FEED CONSUMED	TOTAL MILLIGRAMS THIAMIN CONSUMED	APPROXIMATE THIAMIN INTAKE PER 100 GM. OF FEED
	μg.		*kg.*	*mg.*	*μg.*
2959 [1]	12	37	6.51	3.05	47
2959 [1]	12	21	6.48	3.29	51
3363 [1]	15	71	29.56	16.94	57
2960 [1]	18	35	6.00	4.17	70
2959 [1]	25	21	5.47	4.58	84
2960 [1]	25	44	15.76	11.97	76
2951 [1]	25	49	9.11	8.31	91
3365 [1]	25	71	38.18	29.58	77
3366 [1]	25	71	43.43	31.20	72
2959	37	28	20.22	18.41	91
2960	37	26	21.80	21.45	98
2951	50	28	10.20	14.09	138
3367	50	71	57.34	74.91	131

[1] These animals limited their rate of food consumption presumably because not enough thiamin was fed. The remaining pigs ate all the food placed before them.

If an average is taken of the five values obtained for those pigs receiving thiamin at the 25 μg. level a value of 80 μg. per 100 gm. of air dry feed is obtained. An average of 94 μg. is obtained for those two pigs fed the next highest or 37 μg. level of thiamin fed. Since the pigs on the 25 μg. level refused some feed while those on the 37 μg. level ate all the feed placed before them the actual thiamin requirement appears to be be-

tween these two levels or between 80 and 94 μg. per 100 gm. of feed.

Assuming the diet to contain approximately 75% of carbohydrate and protein the requirement per 100 gm. of carbohydrate and protein can be placed at between 106 and 125 μg.

The above figures are in fairly close agreement with those given by Arnold and Elvehjem ('39) for the thiamin requirement of the dog, Jukes and Heitman ('40) for the thiamin requirement of the chicken, and the value indicated for all species by Williams and Spies ('38). These approximations of the thiamin requirement of pigs hitherto unreported, may be considered as another piece of evidence that the thiamin requirement for all species of animals may be the same when expressed per unit of carbohydrate and protein consumed.

SUMMARY

Thirty-six pigs were fed autoclaved and sodium-sulfite sulfur dioxide treated basal diets with and without a supplement of thiamin. In the preliminary work on the autoclaved diets, animals developed convulsions, locomotor incoordination, paralysis, and other symptoms of malnutrition even when thiamin was given.

Animals on the sodium sulfite-sulfur dioxide treated diets without added thiamin consistently developed symptoms due to a specific lack of thiamin. These symptoms were: almost complete refusal of food with occasional vomiting, extreme emaciation, and marked lowering of body temperature. When thiamin was not added, death usually occurred within 5 weeks. Upon autopsy of these animals a very flabby heart was noted. The intestinal tract was always almost completely empty but pathological changes were not noted in every case. In some cases liver damage was noted.

When enough thiamin was added, an approximately normal rate of growth was maintained with the alleviation of all deficiency symptoms. Attempt has been made to relate data on thiamin requirements to feed intake as well as to body weight. For swine under the experimental conditions described the

supplemental thiamin chloride hydrochloride requirement appears to be between 106 and 120 μg. per 100 gm. of carbohydrate and protein consumed.

Rats fed the same diets as those fed the pigs substantiated the results obtained with the pigs.

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PLATE

PLATE 1

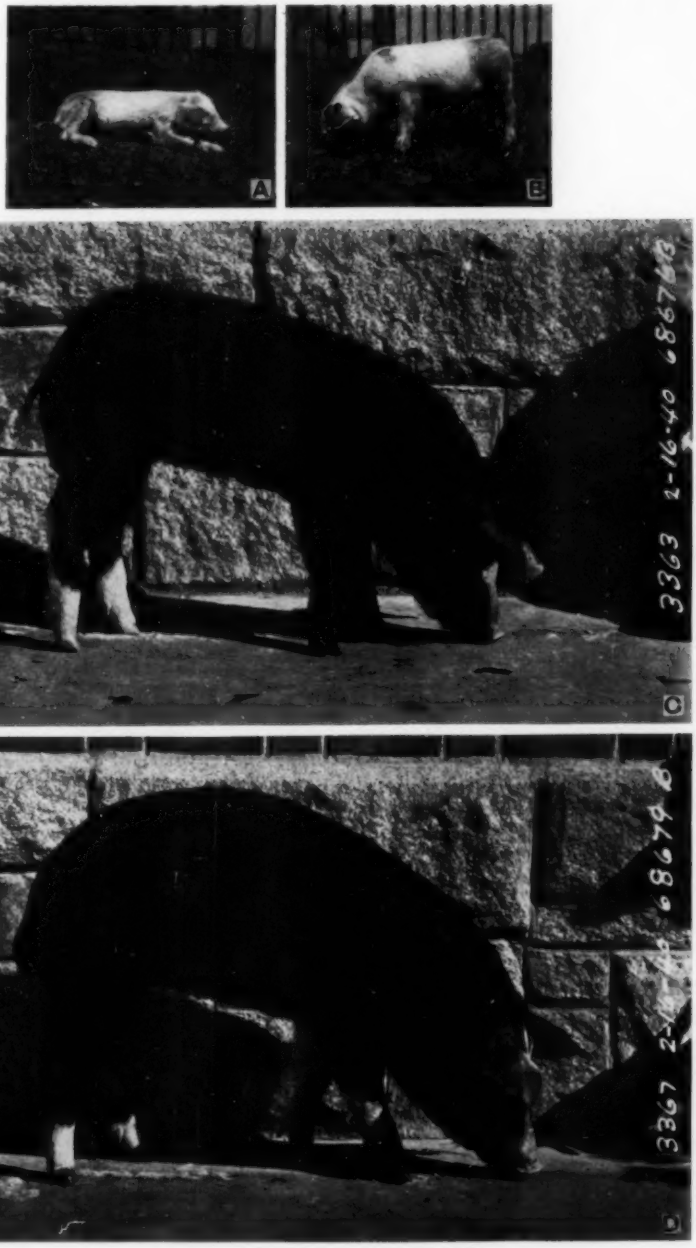
EXPLANATION OF FIGURES

A Pig 2586. Picture taken at 56 days of age after being on the thiamin deficient ration DS5 for 32 days. The animal died from thiamin deficiency the next day after the above picture was taken. Weight 6.07 kg.

B Pig 2587. Litter mate to pig 2586. Picture taken at the same time as that of pig 2586. Weight 14.64 kg. The only difference in the treatment of the two animals was the feeding of 25 μg. of thiamin per kilogram body weight to pig 2587 during the 32-day period preceding the taking of the picture.

C Pig 3363. Picture taken at 99 days of age. This animal had received 15 μg. of thiamin per kilogram body weight for 64 days after a thiamin depletion period of 14 days on diet DS7. Weight, 21.79 kg.

D Pig 3367. Litter mate to pig 3363. Picture taken at the same time as that of pig 3363. Weight 37.8 kg. Pig 3367 received 50 μg. of thiamin per kilogram of body weight as compared to 15 μg. fed pig 3363.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ANTI-GREY HAIR VITAMIN DEFICIENCY IN THE SILVER FOX [1]

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THREE FIGURES

(Received for publication July 22, 1940)

It has been found in this and other laboratories that rats, dogs and guinea pigs (Morgan and Simms, '40) suffer extensive depigmentation of the fur as a result of "filtrate factor" or anti-grey hair vitamin deficiency. When the opportunity [2] to produce a similar deficiency in young silver foxes was offered us we accepted it with interest because of the peculiar importance and variety of fur color in this species. Normally the markings on silver fox fur resemble those of black rats during the earlier stages of the anti-grey vitamin deficiency. It seemed possible that this species might be especially susceptible to the greying deficiency. After our experiment had been begun a somewhat similar study was reported by Lunde and Kringstad ('39). They gave an alkaline autoclaved yeast extract and synthetic vitamin B_1 along with a vitamin B-free basal diet to foxes 11 weeks of age. After 8 to 10 weeks, greying began on the snouts and worked backward toward the eye-sockets. Control animals given the anti-grey substance which they call B_x were of normal color. They mention the work of

[1] This investigation has been aided by a grant from the Josiah Macy, Jr. Foundation. Assistance was rendered also by Works Progress Administration Official Project 665-08-3-30, Unit A-24, assigned to the University of California.

[2] This experiment was made possible through the cooperation of Mr. F. J. Mullins of East Bay Fisheries, Inc. of San Francisco and Mr. Elmer Atkinson, fox breeder of Aptos, California. We gratefully acknowledge their indispensable assistance.

Schoop ('38) who described a "sand coloredness' in newborn foxes which he thought due to lack of vitamin C. Lunde and Kringstad state, however, that they had found the fox not to require vitamin C, an observation confirmed by our experiment. Lunde and Kringstad concluded that the "sand coloredness" must have been caused by anti-grey vitamin deficiency.

EXPERIMENTAL

Six young silver foxes from three litters of highly inbred stock were used in our experiment. They were kept in raised open pens on chicken wire and were never allowed to run in

TABLE 1

Growth of foxes on purified diet

FOX, NUMBER AND SEX	LITTER NUMBER	DIET	WEIGHTS IN KILOGRAMS					
			June 12	July 8	July 22	Sept. 5	Nov. 20	Dec. 11
1, ♀	3	Normal	1.86	2.78	3.17		3.91	4.14
2, ♂	1	Normal	2.53	3.58	4.43		5.40	5.55
3, ♂	2	Normal	2.30	3.83	4.06		5.98	6.18
4, ♀	2	Deficient	2.02	3.13	3.30	3.98	4.40	4.38
5, ♂	1	Deficient	2.41	3.47	3.63	5.45	6.42	6.55
6, ♀	2	Deficient	2.10	2.95	3.24			

the open or to touch the soil. The parents were each fed 12 to 14 ounces daily of a breeding ration made up of 30% fresh meat products, 30% canned whole fish,[3] 40% dry cereal fox feed, and in addition 2 to 3 ounces of carrots and figs. The young were born late in April, 1939 and were weaned the last week in May. The growth of the six animals and the diets to which they were assigned are indicated in table 1.

The basal diet was a modification of that used for dogs in a similar experiment. Its composition, in per cent, was washed casein [4] 30, hydrogenated cottonseed oil [5] 10, salt mixture 2.5,

[3] Silvertone, canned California sardines packed with concentrated fish juices by East Bay Fisheries, Inc.

[4] The washed casein was kindly supplied us by Dr. Paul D. V. Manning of the Western Condensing Company of San Francisco.

[5] Crisco.

wheat germ 15, sucrose 39.5, bone ash 2, agar 1. The wheat germ provided 3.75 mg. thiamin per kilogram of diet and undoubtedly sufficient vitamin B_6. There were added 35 mg. nicotinic acid and 2 mg. riboflavin per kilogram of diet, and each fox was given separately daily 1 teaspoonful (4 gm.) of soup-fin shark liver oil [6] containing 6000 units of vitamin A and 420 units of vitamin D per gram. For three of the animals, nos. 1, 2, and 3, the basal diet was supplemented with 19 cc. per kilogram of yeast filtrate which was known to be rich in the anti-grey hair factor. By actual test with rats each cubic centimeter of the filtrate preparation was found to be equivalent to 1.2 gm. of the yeast from which it was made, thus providing the diet with filtrate potency equivalent to 2% of dried yeast in the ration.

RESULTS

The regular stock ration was gradually replaced by the purified diet over a period of 3 to 4 weeks until by July 1 the animals were receiving practically only the latter. By July 18 the three foxes on deficient diet were rapidly losing fur so that their coats appeared short and cottony like those of very young animals. Their snouts and ears became grey in color. The male, no. 5, developed a dry scaly condition with loss of hair on the tail. On July 26 fox no. 6, which had been active and had maintained food intake as well as any of the others, suddenly died. On the same day an autopsy was performed and tissues saved for histological examination. No evidence of infection or any other abnormality was found except for the presence of a greatly enlarged and hemorrhagic thymus.

Both lots of foxes had eaten the basal diet somewhat unenthusiastically but those on the deficient ration ate considerably less than the others. Beginning August 3 the two surviving deficient animals were given the regular stock ration, 8 to 11 ounces, plus 1 to 3 ounces of the basal diet. Nevertheless they continued to lose fur until the tails were hairless and the skin

[6] Supplied to us by East Bay Fisheries, Inc.

was hard and dry. Beginning August 20 a concentrated rice bran preparation was given, 1 teaspoonful daily per fox, and within 2 weeks a new growth of creamy under-fur began to appear. This under-fur developed rapidly, darkened somewhat but retained an unusual silvery grey color. The guard hairs, particularly on the tails, were slow in appearing and somewhat brownish in color.

Fig. 1 Photographs of foxes fed experimental diets taken during the fur re-growth period in October, 1939. Fox 5 had suffered the anti-grey vitamin deficiency for 44 days, fox 3 had adequate diet.

In the meantime the three animals which had received the filtrate factor-containing diet made excellent growth and did not lose fur at any time. Their under-fur had the usual very dark, almost black, color and the guard hairs were long and dark with somewhat less than the expected silvering. The photographs shown in figure 1 were taken in October.

On December 11, 1939, all five remaining foxes were killed with carbon monoxide gas and pelted. Careful autopsies [7] were made in all cases and tissue specimens saved for study. Thyroids, kidneys, livers, lungs, heart, spleen and sex glands were weighed but no divergence from normal was seen in any of these organs. The only abnormality noted was the presence

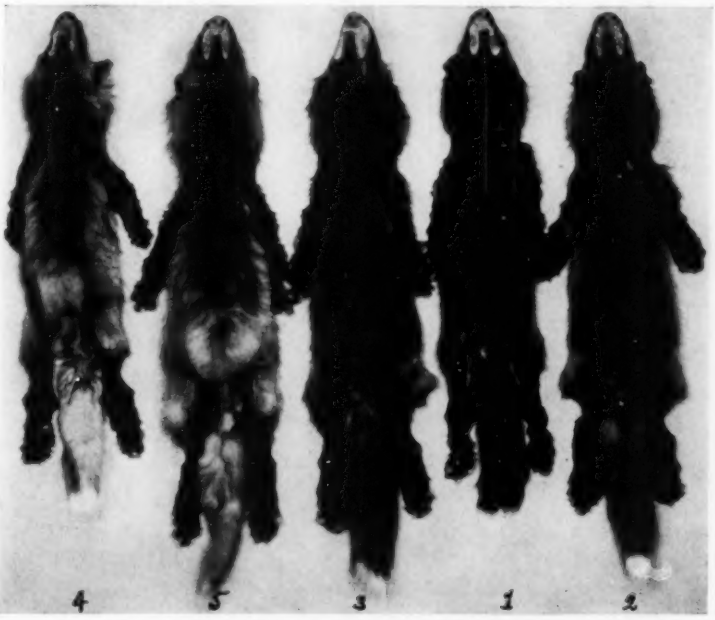

Fig. 2 Front view of pelts of the surviving foxes. Numbers 4 and 5 had anti-grey vitamin deficient diet for 44 days, nos. 1, 2 and 3 had received an adequate diet.

of large red mottled thymuses in the two deficient animals and no trace of persistent thymus in the other three.

Microscopic examination [8] of all the organs revealed no abnormality in any of the hearts, spleens, thyroids, stomachs,

[7] We wish to thank Nobuko Shimotori and Mary Groody who assisted in the autopsies.

[8] We acknowledge the invaluable assistance of Dr. D. A. Singman of the Pathology Division, University of California Medical School, in the study of these tissues.

testes or ovaries. Mild passive congestion was found in the lungs and mild or moderate fatty infiltration in livers and in renal tubules of all the animals killed with carbon monoxide. These changes are to be expected in asphyxia. No evidences of infection were found. The surviving thymuses of the three

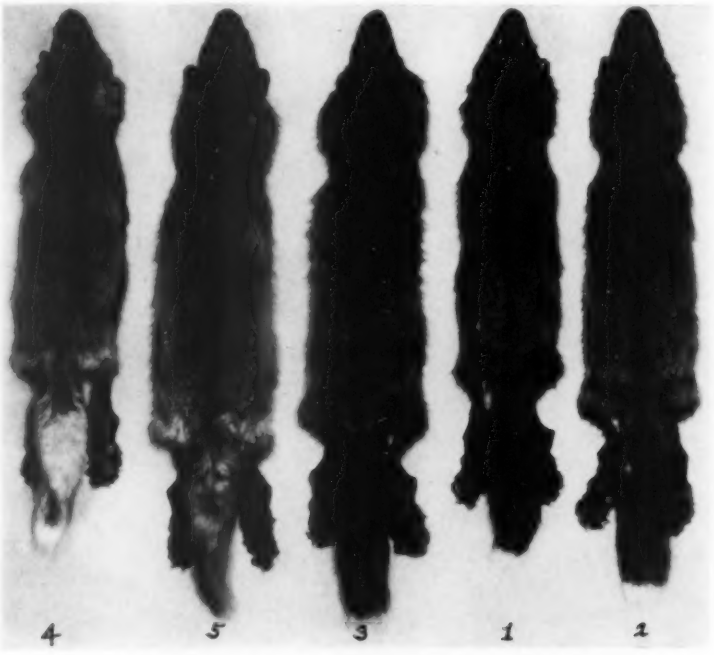

Fig. 3 Back view of pelts of surviving foxes.

deficient animals were mottled with surface hemorrhages, that of no. 6, which died early more definitely than the others. None of them showed any evidence of involution.

Slight abnormality of the adrenals occurred in the deficient animals, some deeply pigmented cells being present in the medulla and vascular and partly degenerated areas in the reticularis at the polar ends of the cortex next to the medulla,

but the condition was not as pronounced as that we have seen in numerous rats which had become grey from the same deficiency.

The pelts were photographed as soon as possible after their removal and are shown in figures 2 and 3. Fox no. 4 was most depigmented with completely grey brush and both 4 and 5 had white belly fur, a phenomenon not usually seen even in the lightest colored silver foxes.

DISCUSSION

The Norway platinum foxes recently obtained as mutants as described by Mohr and Tuff ('39) have distinct white markings on the face, legs, neck and belly as well as only partly pigmented under-fur and guard hairs. These markings appear to be inherited as a dominant characteristic in heterozygous individuals. Whether long-continued slight anti-grey vitamin deficiency may be involved in the production of such mutants is an interesting question. Certainly these silver foxes were more susceptible to the depigmenting deficiency than the rats and dogs previously studied in this laboratory.

The most outstanding feature of this experiment was the early and devastating effect upon the fur of the deprivation of filtrate factors even though this deprivation was only partial. The diet contained 15% of whole wheat germ which was included chiefly to provide vitamin E but which was by no means devoid of the filtrate factors. Nevertheless after only 26 days on this regime one of the foxes died apparently of the deficiency, and although the two remaining animals were changed at once to the complete stock diet and were given additional doses of rice bran concentrate their fur was lost and the new fur which appeared was largely depigmented. The survival of thymus which was red-pigmented in spots and without evidence of involution in each of the three animals on the deficient diet is another important observation. This thymus survival may be connected with the slight but unmistakable damage to the adrenal cortex seen in the same animals. It has been shown by Crede and Moon ('40) that adrenocorticotropic extracts exert

a thymus destructive effect in normal and castrate but not in adrenalectomized rats. The adrenal cortical secretion would therefore appear to be at least partially responsible for the normal disappearance of the thymus. Abnormal survival of the thymus noted in the experimental foxes may perhaps be ascribed to partial failure of adrenal cortex influence.

McKibbin, Madden, Black and Elvehjem ('39) found "a peculiar red pigment spotted appearance of the thymus gland" in two dogs which had died after relatively short periods on filtrate-factor deficient diet. The ages of these dogs were not given but apparently they were young, supposedly 2 to 3 months old if they were placed on the diet at weaning. They lived only 20 and 30 days after the supplementary feeding began, a much shorter survival than we have usually found with filtrate-factor deficient dogs. Fouts, Helmer and Lepkovsky ('40) who used adult dogs likewise noted longer survival periods. Thymus survival might be expected in dogs of the age mentioned but apparently our normal foxes nos. 1, 2 and 3, 7 to 8 months of age, had lost so much of the thymus as to make it impossible to find the remnants although they were carefully sought.

The question arises as to whether decreased adrenal cortex activity was the primary result of the filtrate factor deficiency with hypertrophied thymus resulting therefrom as noted in Addison's disease (Rowntree, '35), or whether the thymus is primarily affected. There may be in this a clue to the mysterious role of the thymus and the clinical condition known as "status thymicolymphaticus."

SUMMARY

Six young silver foxes of three litters were placed on purified diet supplemented by fish liver oil, wheat germ, riboflavin and nicotinic acid, three receiving also a yeast "filtrate factor" preparation. One of those which did not receive filtrate factor died after 26 days and the other two had lost most of their fur although they had been left on the deficient diet only 44 days. Their new fur was light grey in color.

On autopsy of the fox that died and the other two on deficient diet pelted 4 months later large red-mottled thymuses were found.

The three foxes which received the filtrate factor retained their fur, had no surviving thymuses and had normal dark silver pelts.

These observations are similar to those previously observed in rats and dogs and point to possible interrelation of nutritional and endocrine mechanisms.

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MEAD JOHNSON & COMPANY 'B-COMPLEX' AWARD

Nominations are solicited for the 1941 Award of $1,000 established by Mead Johnson and Company to promote researches dealing with the B complex vitamins. The recipient of this Award will be chosen by a Committee of Judges of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute at Chicago on April 16, 1941.

The Award will be given to the laboratory (non-clinical) or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year January 1st to December 31st the most meritorious scientific report dealing with the field of the 'B-complex' vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the prize be divided between two or more parties. It may also be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Committee of Judges, nominations for this award for work published in 1940 must be in the hands of the Secretary by January 25, 1941. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Committee of Judges in its consideration of the nomination.

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SECRETARY, AMERICAN INSTITUTE OF NUTRITION

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ERRATUM

In table 1, page 519 of the paper "Fasting catabolism and food utilization of calcium-deficient rats," by Max Kleiber, Muriel D. D. Boelter and David M. Greenberg, which appeared in The Journal of Nutrition, vol. 19, no. 6, June, 1940, the synthetic vitamin supplements should be per kilo of diet; namely Thiamin Chloride 5.0 mg. synthetic riboflavin 4.0 mg., and nicotinic acid 50.0 mg. per *kilo of diet.*

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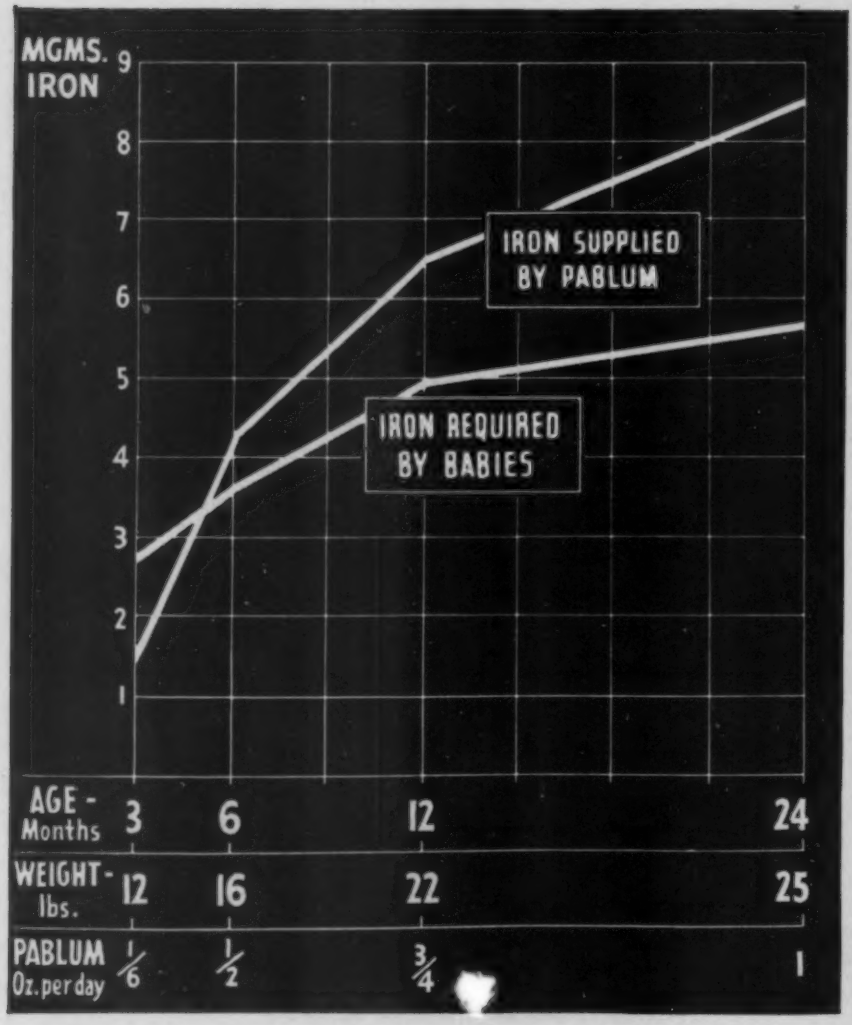

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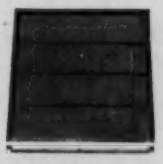

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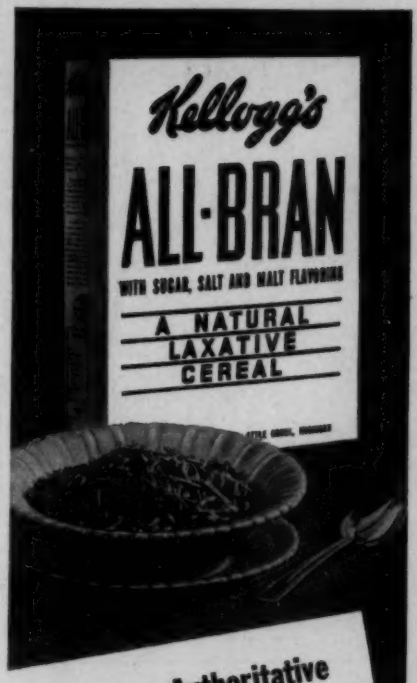

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